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Article

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From the Promiscuous Asenapine to Potent Fluorescent Ligands Acting at a Series of Aminergic G Protein Coupled Receptors.

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ABSTRACT:

Monoamine neurotransmitters such as serotonin, dopamine, histamine and noradrenaline have important and varied physiological functions and similar chemical structures. Representing important pharmaceutical drug targets, the corresponding G protein coupled receptors (termed aminergic GPCRs) belong to the class of cell membrane receptors and share many levels of similarity as well. Given their pharmacological and structural closeness, one could hypothesize the possibility to derivatize a ubiquitous ligand to afford rapidly fluorescent probes for a large set of GPCRs to be used for instance in FRET-based binding assays. Here we report fluorescent derivatives of the non-selective agent asenapine which were designed, synthesized and evaluated as ligands of 34 serotonin, dopamine, histamine, melatonin, acetylcholine and adrenergic receptors. It appears that this strategy led rapidly to the discovery and development of nanomolar affinity fluorescent probes for 14 aminergic GPCRs. Selected probes were tested in competition binding assays with unlabelled competitors in order to demonstrate their suitability for drug discovery purposes.

INTRODUCTION

With seven hydrophobic transmembrane (TM) α-helical segments as a signature feature, G protein-coupled receptors (GPCRs) represent the largest class of membrane proteins in the human genome.[1] Because they are signaling transducers (transmission belts) involved in many physiological processes,[2] GPCRs are associated with various human diseases,[3] have many therapeutic applications and represent targets of great importance for the pharmaceutical industry.[4]

As such, targets from the GPCR superfamily have generated around 30% of the currently marketed drugs.[5] Actually, most of these targets belong to the family of Class A (or rhodopsin-like) GPCRs[6] and more precisely to the subfamily of aminergic GPCRs which account for only 10 % of the entire family.[7]

This pharmaceutical bias toward aminergic GPCRs lies on two main reasons. A historical one at first, since aminergic neurotransmitters were among first to be isolated and identified from biological fluids and tissues. Furthermore the aminergic β_2-adrenergic GPCR was the first GPCR ever sequenced after the atypical rhodopsine and many homologous aminergic receptors were then sequenced as well.[8] As a consequence aminergic GPCRs have been extensively investigated along with the development of the GPCR research field and drug discovery since the second half of the previous century. They are considered as the best understood family of GPCRs. In second the multi-level similarity inside the subfamily: a) the endogenous ligands are biogenic amines neurotransmitters and derivatives (acetylcholine, serotonin, dopamine, histamine, noradrenaline, melatonin, etc.) with strikingly close chemical structures; b) in sequence alignment evaluations, orthosteric binding pockets keep similar ligand binding determinants (key ligand binding residues);[9] c) tertiary structures are well conserved despite amino acid diversity as shown by early modelling studies.[10,11] Indeed, orthosteric binding pockets consist of cavities similarly located between extracellular loops

and the upper side of the transmembrane region. The position is conserved but there is diversity in its shape, its depth and its aminoacid composition.[12] Therefore aminergic GPCRs display high levels of pharmacological similarity. As a consequence, aminergic GPCRs have also numerous small molecule ligands with polypharmacology and high chemical resemblance.

Indeed, in the recent years, many high profile studies regarding class A GPCRs have emerged in the literature. Paolini *et al.* performed a mapping of pharmacological target space and illustrated the high target promiscuity of aminergic GPCRs.[13] Keiser *et al.* quantitatively grouped and related proteins based on the chemical similarity of their ligands.[14] Later, off-target effects were experimentally predicted and demonstrated for FDA-approved and investigational drugs.[15] Other similar publications illustrating cross-interactions of GPCR ligands are also available.[16-21] In a functional signaling perspective, Wichard *et al.* identified amino acid positions governing antagonistic and agonistic effects for class A GPCRs using the Mutual Information analysis.[22] Using comparative sequence-based and ligand-based classifications for aminergic GPCRs, van der Horst *et al.* reached nearly identical images.[23] This work was later confirmed by Lin *et al.* and even included non-GPCR targets.[24] More recently, Kooistra *et al.* studied the molecular determinants of ligand binding to aminergic GPCRs with histamine receptors as reference receptors.[20]

In this flourishing context, it is crystal clear that accurate pharmacological data and further, high quality pharmacological assays are critical for chemogenomics studies.[25] As such, ligand binding assays represent an important frontline.[26] In the recent years, traditional radioligand binding assays have been slowly replaced with fluorescence-based ligand binding assays.[27-31] Based on small-molecule (peptidic and nonpeptidic) fluorescent conjugates, a growing toolbox is taking shape, especially regarding class A GPCRs, which has been successfully applied in techniques such as flow cytometry, fluorescence correlation spectroscopy,

fluorescence microscopy, fluorescence polarization, fluorescence resonance energy transfer, and scanning confocal microscopy.[32] Among these, time-resolved fluorescence resonance energy ransfer (TR-FRET) is particularly gaining momentum.[33] Rare-earth lanthanides with long emission half-lives as donor fluorophores in combination with suitable acceptor probes and time resolved readouts have enabled significant improvements in signal-to-noise ratios, fastness, sensitivity, stability, reliability, robustness and compatibility with high throughput screening (HTS) assays on GPCRs, in comparison with other fluorescence readouts.[34] This approach based on homogeneous time resolved fluorescence (separation is no longer required for bound and free fluorescent probes) is being strengthened by recent successes.[29,35-37]

In order to investigate GPCR binding properties and oligomerization, fluorescent ligands have been reported for targets such as oxytocin and vasopressin receptors,[38] the parathyroid hormone receptor,[39] the growth hormone secretagogue receptor type 1a,[40] the protease activated receptor 2,[41] the human complement 5a receptor[42] and chemokine receptors.[43] However, the number and profile diversity of GPCR fluorescent ligands need to be extended.

As such, the development and the evaluation of FRET-based binding assays for a large number of GPCRs remain of great interest for understanding the fundamental mechanisms of action of GPCRs such as homo- and heterodimerization or allostery and for the drug discovery field. As for drug design and development, it also represents quite a difficult challenge if one attempts to develop hundreds of fluorescent ligands to target *specifically* their hundreds of GPCRs. However, we reasoned that in order to obtain a specific FRET-based binding assay, the fluorescent ligand does not need to be specific, the signal does, and the specificity of the signal will arise from the specific expression of the tagged, donor target receptor in the cell assay system. Therefore, the original concept we developed was to identify and tag a promiscuous GPCR ligand (frequent GPCR hitter) to attempt turning it into a limited series of fluorescent ligands binding to a large number of GPCRs and thus providing

specific time resolved FRET-based binding assays and molecular probes to investigate ligand kinetic parameters for applications in basic and applied research.

RESULTS

Design

Intending to double down on their multi-level similarity, we hypothesized that, as similar receptors bind similar ligands, non-selective "universal" fluorescent probes should be possible to find for aminergic GPCRs. Asenapine, a psychopharmacologic agent currently in clinical use for the treatment of acute schizophrenia and bipolar disorders (Figure 1), was suitable in many aspects.[44] Indeed asenapine has subnanomolar and nanomolar affinities ($K_i \approx 5$ nM) for diverse and numerous subtypes of aminergic GPCRs (serotonin, norepinephrine, dopamine and histamine receptors).[45] In contrast, it was described with micromolar affinities for muscarinic acetylcholine receptors and melatonin receptors in addition to no detectable affinity for histamine H_3 and H_4 receptors which could all serve as negative controls. In addition, asenapine proved clear antagonistic properties in functional assays for all its targets, a stable behavior highly appreciable for a platform. Asenapine displayed the classical pharmacophore of biogenic amine receptors: a protonable nitrogen, likely to make a salt bridge with the conserved aspartate residue on TM 3, and aromatic rings at a 5 to 6 Å distance, probably embedded against TM 5 and 6. Such ligands can usually be branched on the nitrogen atom with different substituents in retaining most of the time some level of biological activity. However a minimum length of approximately 15 atoms is usually required from the linker to maintain the hindering fluorophore at the mouth of the binding cleft in order to prevent any detrimental interaction with the target receptor.[31] For example, as shown in previous works,[28-31, 35-40] fluorescent groups can be grafted on linkers of varied lengths and

chemical nature. Herein we decided to remove the N-methyl group of asenapine (Scheme 1) and to replace it with a linker carrying the fluorophore (Figure 1).

Four different linkers and two fluorophores were combined. Linkers with different length and hydrophobicity included a 3 atom aminoethyl spacer ('short linker', SL), a 16 atom polymethylenic monoamide spacer ('long liker, LL), a corresponding 15 atom pegylated spacer '(pegylated linker', PL) and a 16 atom, polymethylenic diamide spacer ('very long linker', VLL; Scheme 2). These linkers were connected to norasenapine (Schemes 3 and 4) and coupled via an amide bond to two structurally different fluorophores that were already known to be compatible with time resolved FRET: the cyanine derivative 2-((1E,3E)-5-((Z)-3-(4-((2,5-dioxopyrrolidin-1-yl)oxy)-4-oxobutyl)-1-ethyl-3-methyl-5-sulfoindolin-2-ylidene)penta-1,3-dien-1-yl)-1-ethyl-3,3-dimethyl-3H-indol-1-ium-5-sulfonate (DY647; D) and the fluorescein derivative, 6-(fluorescein-5-carboxamido) hexanoic acid (5-SFX; S, Scheme 4).[39] Fluorescein itself (FLUO; F) was combined with the diamido spacer only. Nine fluorescent derivatives of asenapine have thus been prepared and evaluated in TR-FRET ligand binding assays regarding serotonin, dopamine, histamine, melatonin, acetylcholine and adrenergic receptors.

Chemistry

Norasenapine. Supply of norasenapine **17** was secured from commercially available asenapinium maleate **14** (racemate) in 71% overall yield. Treatment of **14** with polymer linked 1,5,7-triazabicyclo[4.4.0]dec-5-ene (PL-TBD) led to free asenapine **15** which could be converted into norasenapine **17** by reaction with 1-chloroethyl chloroformate in 1,2-dichloroethane followed by treatment with methanol according to Olofson and Senet (Scheme 1).[46]

Linkers. Preparation of mesylate **4** started by selective monotosylation of diol **1** with tosyl

chloride, silver(I) oxide and potassium iodide.[47] It was completed by reaction of tosylate **2** with sodium azide to give **3** and by mesylation to provide compound **4** (Scheme 2; 60% overall yield).[48]

The synthesis of aldehyde **7** was first attempted by treatment of commercially available acid **5** with allylamine followed by oxidation with $RuCl_3$-$NaIO_4$. However, the reaction was sluggish and **7** was only obtained in moderate yield. As an alternative, acid **5** was converted into amide **6** with 3-aminopropane-1,2-diol under benzotriazol-1-yl-oxytripyrrolidinophosphonium hexafluorophosphate (PyBOP) activation and then into **7** by oxidative cleavage with sodium periodate in 93% overall yield (Scheme 2). The same reagents also allowed the preparation of aldehyde **13** from acid **11**. The latter compound was obtained by reaction of methyl 5-aminopentanoate **8** with protected *N*-succinimidyl 6-aminohexanoate **9** followed by saponification of the methyl ester **10**. The yield for the conversion of **8** into **13** was 71% (Scheme 2).

Fluorescent asenapine derivatives. The nine ligands **26-34** have been obtained by connecting norasenapine **17** to the dyes DY467, 5-SFX or fluorescein with proper linkers (Scheme 3). To this end, we have prepared amines **19**, **21**, and **25** from *N*-Boc-2-aminoacetaldehyde, aldehyde **7**, and aldehyde **13** respectively, by reductive amination with norasenapine **17** followed by amine deprotection with trifluoroacetic acid. We have also prepared amine **23** by reaction of norasenapine **17** with mesylate **4** followed by a Staudinger reduction of azide **22** with polymer supported triphenylphosphine (PS-PPh_3) in THF-water. Reaction of amines **19**, **21**, **23**, and **25** with the *N*-succinimidyl esters of DY647 and 5-SFX led to the ligands **26-33** (Scheme 4). Ligand **34** was prepared in the same way by labelling amine **25** with the activated ester of fluorescein. The fluorescence properties of the ligands **26-34** are reported in the experimental part and in the Supporting Information part.

Saturation binding experiments

TR-FRET receptor binding assays were performed to evaluate the binding affinities of the nine fluorescent probes **26** to **34** on 12 serotonin, 5 dopamine, 4 histamine, 5 acetylcholine and 2 melatonin receptor subtypes. As already described,[35] the transient expression of the target proteins as SNAP-tagged constructs at the surface of HEK293T cells was achieved. Reactions with SNAP substrates resulted in SNAP-tagged constructs wearing time-resolved FRET donors of fluorescence (Tb cryptate). Hence, saturation experiments were performed with increasing concentrations of probes **26-34**. Nonspecific signals were obtained with excess of unlabeled compound (10 μM asenapine) and specific signals were then plotted as functions of fluorescent probes concentrations in order to give dissociation constants (K_d). All results are presented below with a threshold for K_d values at 1 μM (see Tables 1-4) and illustrated with compound **31** in Figure 2. All results are also presented with the corresponding signal-to-noise (S/N) ratios calculated in the binding assay as the ratio of the total signal on the nonspecific signal at the K_d value. The threshold for the S/N ratios was set at 2 meaning that specific signal should amount to one half of the total signal. The results from all ligand binding assays are summarized in Table 5 where the number of hit aminergic GPCRs is indicated for each fluorescent ligand. The binding profile of the different probes is illustrated in Figure 3.

Overall, as expected, it appears that the fluorescent tracers bound the same subtypes of aminergic GPCRs as the parent asenapine with nanomolar range affinities (see Tables 1-4).

Results with serotonin receptors (Table 1). On $5HT_{1a}$ receptors, compound **33** shows an affinity (K_d = 3 nM) comparable to asenapine (K_d = 2.5 nM) while compounds **30** and **31** display a 10 fold lower affinity. All other asenapine derivatives are inactive suggesting that the combination of the polymethylenic diamide spacer and DY647 fluorophore is ideal and unique in providing a high affinity fluorescent probe for the $5HT_{1a}$ receptor. The pegylated

spacer is also accepted by the receptor, the fluorophore making no difference in this case (see compounds **30** and **31**; K_d = 31 nM and K_d = 32 nM, respectively).

On the $5HT_{1b}$ receptor, the results are quite different. The 5-SFX short chain compound **26** shows some significant affinity (K_d = 169 nM) while the DY647 analogue is inactive. The reverse is observed for the long chain polymethylenic monoamide derivatives **28** and **29**: the DY647 compound (**29**; K_d = 63 nM) is the most potent fluorescent ligand of this receptor whereas its 5-SFX analogue **28** is inactive. Interestingly, the pegylated homologues **30** and **31** are inactive while an intermediate affinity is recovered in the diamide linker series (**32**, **33**; K_d = 121 nM and 106 nM, respectively) with a much less pronounced discriminating effect of these two fluorophores and a detrimental effect of fluorescein (**34**; K_d > 1000 nM).

On the $5HT_{1d}$ receptor, the short spacer derivatives **26** and **27** are inactive. Asenapine derivatives with longer linkers show a significant affinity ranging from 17 nM (compound **31**) to the 50 nM range (**28**, **29**, **30**, **33)**. The choice of the fluorophore is discriminant again but only in the diamide series since **32** and **34** are completely inactive.

On the $5HT_{1e}$ and $5HT_{1f}$ receptors, no significantly active fluorescent probe was identified with the exception of compound **26** which displays affinity in the low micromolar range for $5HT_{1f}$ (K_d = 656 nM). To our knowledge, no affinity data for asenapine has been reported on these receptors.

On the $5HT_{2a}$ receptor, affinity is restricted to probes with the polymethylenic monoamide spacer and a preference for the DY467 fluorophore (**30**, K_d = 88 nM; **31**, K_d = 17 nM).

On the $5HT_{2b}$, the activity profile of the series of probes is again different. The short linker series is inactive. In the long linker series, only the DY647 derivative **29** shows affinity (K_d = 40 nM) in the monoamide series, while all compounds of the diamide series (**32** to **34**) are inactive. Both molecules carrying the pegylated linker provide high affinity probes with a

preference towards the DY647 fluorophore since compound **31** alone reaches an 8 nM affinity.

Binding to the $5HT_{2c}$ receptor seems less stringent. All probes bind at sub-micromolar concentrations with the exception of the short-chain DY647 derivative **27** which is inactive while its 5-SFX analogue **26** is very potent with a 20 nM affinity. Thus nanomolar fluorescent probes could be identified for the $5HT_{2a\text{-}c}$ receptors however with a one to two log decrease in affinity compared to the parent compound, asenapine.

Interestingly, none of the probes display affinity for the $5HT_4$ and $5HT_{5a}$ receptors. In contrast, for the $5HT_6$ receptor, probes **26**, **29** and **33** show a reasonably high affinity with K_d = 49 nM, 122 nM and 55 nM, respectively, while all other probes are inactive. Again, the 5-SFX fluorophore was preferred on short chain derivative **26** whereas DY647 provides more active compounds on the polymethylenic long chain derivatives **29** and **33**. The pegylated spacer is unfavorable to activity on this receptor (**30**, **31**). In contrast, on the $5HT_7$ receptor subtype, the pegylated compound **31** is the most potent (K_d = 36 nM) while compound **29** was the only other active one with an affinity in the same range (K_d = 47 nM).

Results with dopamine receptors (Table 2). The affinity of the nine probes was determined on the five dopamine receptor subtypes. On dopamine D_1 receptor, only compound **26** carrying the short spacer and the 5-SFX fluorophore displays a significant affinity for the receptor (K_d = 60 nM). The same compound has a noticeable 6 nM affinity for the D_2 receptor. If one excludes its DY647 analogue **27**, all other probes are active in the 13-146 nM range with a predominant efficacy of the DY647 analogues **31** and **33**. The contribution to efficacy of the two fluorophores is opposite in the short chain and the long chain series.

On the D_3 receptor, a similar pattern is observed. The most active probes are again **26**, **31** and **33** with K_d = 20 nM, 24 nM and 15 nM, respectively. The difference in affinity compared to homologues with other fluorophores is more significant than on D_2 receptor subtype.

On the D_4 receptor, two compounds with different spacer lengths and fluorophores, namely **26** and **31,** are the only one showing a significant affinity, with K_d = 40 nM and 56 nM, respectively. Finally, none of the nine probes display affinity for the D_5 receptor subtype.

Results with histamine receptors (Table 2). Results on the histamine receptors are clearcut. All probes show a good affinity for the H_1 receptor subtype with K_d ranging from 20 nM to 316 nM. As for several other receptors, the 5-SFX fluorophore is the most favorable on the short spacer while the DY647 is preferred on the long spacer. In deep contrast, all probes are weakly active or inactive on the H_2, H_3 and H_4 receptor subtypes.

Results with the adrenergic receptors (Table 3). On the α_{1a} receptor, five probes show a significant affinity with K_d ranging from 121 to 308 nM. However, the interpretation of the results is quite challenging: the 5-SFX probe **26** is active in the short spacer series (K_d = 308 nM) while its DY647 analogue **27** is inactive; both fluorophores provide activity in the polymethylenic monoamide series (compounds **28** and **29;** K_d = 174 nM and K_d = 121nM, respectively); the DY647 derivative **31** only is active in the pegylated series (K_d = 247 nM); and only the 5-SFX compound **32** is active in the polymethylenic diamide series (K_d = 131 nM). On the α_{1b} receptor, the situation is more clearcut: two DY647 derivatives with long spacers of different nature, namely **29** and **31**, show high potency (K_d = 70 nM and K_d = 2.4 nM, respectively). None of the nine probes bind to the α_{1d} and α_{2a} receptor subtypes. Seven probes present some affinity for the α_{2b} subtype, the best one reaching a 72 nM affinity (compound **33**). Noteworthy, this receptor is the only one in the series accepting a fluorescein tagged ligand with a significant affinity (compound **34**; K_d = 180 nM). Finally, the α_{2c} receptor subtype binds to only one probe (**29**; K_d = 160 nM) with a long polymethylenic monoamide spacer and the DY647 fluorophore. To our knowledge, the affinity of the parent compound, asenapine, for these adrenergic receptors had never been published before. High to

moderate affinity fluorescent probes derived from asenapine could be identified for four adrenergic receptor subtypes out of the six studied here.

Results with the muscarinic and melatonin receptors (Table 4). Asenapine has no affinity for muscarinic receptors.[45] None of the nine fluorescent probes bind to any of the five muscarinic receptor subtypes. None of them binds neither to the MTN_{1A} nor MTN_{1B} melatonin receptors.

Competition binding experiments

As proof of concept, four probes were used to develop novel TR-FRET binding assays for 5 different receptors, namely $5HT_{2b}$, $5HT_{1d}$, $5HT_{2c}$, $5HT_6$ and D_4 receptors. As already described,[36] the target proteins were transiently expressed as SNAP-tagged constructs at the surface of HEK293T cells. In second, reactions with SNAP fluorescent substrates resulted in SNAP-tagged constructs wearing TR-FRET donors of fluorescence (Tb cryptate). The fluorescent probes **26**, **29**, **30** and **31** were added at fixed concentration, at their K_d values. Increasing concentrations of reference competitors were then used to afford inhibition constants that were compared to literature values measured in classical radioligand assays. Results are listed in Table 6 and exemplified in Figure 2 for compound **31**. There is generally a good agreement between both data sets with however a significant shift observed in the particular case of ligand **26** at the D_4 dopamine receptor, when roxindole was used as competitor, and at the $5HT_6$ receptor. It is noteworthy that all the competitions with the reference ligands have led to a total displacement of the tracer and all data can easily be fitted when considering a slope of 1. This suggest the absence of positive or negative binding of the reference ligands when using the fluorescent ligands as tracers.

DISCUSSION

Most fluorescent probes derived from asenapine retained the fluorescence properties of the dye building blocks. They showed high binding affinities for one or several GPCRs. In many cases, the large structural modification introduced on the amino group of asenapine in replacement of the methyl group remarkably allowed retaining most affinity values. Hence, moderate to high affinity fluorescent ligands were discovered for the $5HT_{1a}$, $5HT_{1b}$, $5HT_{1d}$, $5HT_{2a}$, $5HT_{2b}$, $5HT_{2c}$, $5HT_6$, $5HT_7$ serotonin receptors (Table 1); D_1, D_2, D_3 and D_4 dopamine receptors (Table 2); H_1 and H_2 histamine receptors (Table 2); α_{1A}, α_{1B}, α_{2B} and α_{2C} adrenergic receptors (Table 3). Since asenapine had no affinity for histamine H_3 and H_4, muscarinic acetylcholine and melatonin receptors, it is not surprising to find that asenapine-derived fluorescent probes have no significant affinity for this subset of GPCRs (K_d values > 1 μM). Further, no tracers were found for receptors with no published affinity data for asenapine ($5HT_{1e}$, $5HT_{1f}$, $5HT_4$, $5HT_{5a}$, D_5, α_{1D} and α_{2A}) as their K_d values were all over 600 nM. These results illustrate how relevant were the choice of asenapine as platform and the choice of the tagging position on asenapine since fluorescent derivatives, altogether, reproduced the binding profile of the initial chemical platform. Noteworthy, the fluorescence properties of the different ligands are similar to those of the dyes (see experimental part and supplementary material) and were not significantly affected by the shorter and longer linkers.

From the ligand point of view, compound **26** is excellent (K_d <100 nM) for all dopamine receptors except the D_5 subtype. It also shows good affinity for the H_1 histamine (K_d = 42 nM), $5HT_{2C}$ (K_d = 20 nM) and $5HT_6$ (K_d = 49 nM) serotonin receptors but marginal or no affinity for other GPCRs. Interestingly, tracer **27** is very specific since it does not bind any of the 33 GPCR tested except the H_1R (K_d = 105 nM). Compound **34** is also peculiar in that it binds significantly only one 5-HT receptor subtype out of twelve (K_d = 104 nM, $5\text{-}HT_{2C}$ receptor), one dopamine receptor subtype out of five (K_d = 105 nM, D_2 receptor), one histamine receptor subtype out of four (K_d = 118 nM, H_1 receptor) and one adrenaline

receptor subtype out of six (K_d = 180 nM, α_{2B} receptor). All other compounds are more ubiquitous, hitting 7 to 14 receptors out of 27 if one exclude muscarinic and melatonin receptors for which no ligands were found.

Few unexpected results are obtained. It is noteworthy that: a) Tracers with nanomolar range affinities (2.4 nM – 70 nM) are found for $5HT_{1d}$ and α_{1B}, two receptors with no published affinity data for asenapine; b) No tracers are found for GPCRs such as $5\text{-}HT_{5A}$ and α_{2A} with reported high affinities for asenapine.[45]

The length of the linkers as well as the nature of the linkers and of the fluorophores have clearly a strong impact on the affinity and selectivity of the probes. However, it appears very difficult to rationalize these effects. The comparison of probes **26** (12 GPCR hits) and **27** (1 GPCR hit) indicates that the 5-SFX dye performs better than the DY-647 dye when short linkers are used (Table 5). However, longer linkers as in **29**, **31** and **32** provide broad spectrum, potent fluorescent probes as well. Pegylated spacers seem to be slightly more favorable than polymethylene spacers but not dramatically and not systematically (see **28**, **30**, **32**). Finally, the nature of the fluorophore is clearly important to retain affinity. On short linkers as in **26** and **27**, the 5-SFX fluorescein moiety is clearly preferred to the cyanine fluorophore of DY647. However, the reverse is observed on longer chains such as in **28** and **29** while there is no clear difference between **30** and **31** or **32** and **33**. Thus the choice of the fluorophore and of the spacer makes a huge impact on the ability of the ligands to interact with the receptors. One may hypothesize that their asenapine fragment binds in the same orthosteric pocket as asenapine itself while the fluorophore may interact with the loops at the entrance of the binding cleft, in allosteric sites more or less well structured, as already proposed.[30] This would probably affect several properties of the ligands such as binding kinetics and functional efficacy. This may explain the shift in competition assays as seen with compound **26** at the D_4 and $5HT_6$ receptors (Table 6). In addition, the impact on functionality

of the derivatization of asenapine with linkers and fluorophores has not yet been studied. Though unlikely, one may not exclude shifts from antagonism to partial agonism or inverse agonism. Such modifications would not alter the interest of the new probes for competition binding experiments in search for receptor ligands but it should be studied in details if one want to use them for instance for receptor trafficking or receptor binding kinetics studies. Considering the subtlety of our results, the flexibility of the linkers and the limited knowledge of the receptor dynamics in the extracellular loops regions, we considered that the rationalization of our binding data by docking studies could only be achieved by an extensive modeling study coupled to experimental validation by co-crystallization, site directed mutagenesis, kinetic and functional studies or other approaches. This work will be undertaken and reported elsewhere.

In terms of FRET efficacy, S/N ratios significantly vary according to the fluorescent ligand/ receptor pairs. These ratios are dependent (i) on the nature of the fluorophore acceptor (fluorescein *vs* DY647), the fluorophore donor (the Terbium cryptate) being always the same; (ii) on the distance between the two fluorophores when the fluorescent ligand is bound to the receptor. It is noteworthy that in time-resolved FRET, the FRET efficacy is weakly dependent on the relative orientation of the fluorophores. The maximum of S/N equal to 125 has been obtained for probe **24** on dopamine D_3 receptor. S/N ratios greater than 3 are often considered as relevant for time-resolved FRET experiments. Here, in the saturation or competition binding experiments, the assays are particularly sensitive and we observed that a S/N ratio of 2 is relevant to get reliable data. This has been firmly established in using some of them in competition assays with reference compounds for a subset of studied receptors (Table 6).

CONCLUSION

In conclusion, asenapine proved to be indeed a very relevant platform to produce high affinity fluorescent probes for a large set of GPCRs. Starting from this single broad spectrum, nanomolar ligand of a large set of GPCRs, we were able to design a small series of fluorescent probes that hit at least one target GPCR. Some of them hit up to 14 receptors out of 34 tested whereas other probes happened to be rather specific of one of them. Without surprise, the interaction between such probes and a GPCR is very subtle. As known, adding a 'linker' or a fluorescent 'tag' on a ligand must not be considered as neutral or trivial. The length of the linker, its chemical and structural properties, the structure and properties of the fluorophore clearly affect the binding process of the probe in a way that will be difficult to rationalize and to anticipate as indicated by our results.

Although no 'universal' fluorescent probe has been identified, our concept of derivatizing a broad spectrum, high affinity GPCR ligand was quite efficient since a series of 9 probes tagging 14 different class A GPCRs has been rapidly produced. Some of them may represent specific imaging tools (eg compound **27** for H1 receptors), all of them are likely to give access to specific time resolved FRET binding assays with applications in basic research[29] or in drug development.[35]

EXPERIMENTAL SECTION

Chemistry

General methods. Reagents were obtained from commercial sources and used without further purification. DY647-NHS, 5-SDX-NHS and Fluorescein-NHS were purchased from Dyomics GmbH (Jena, Germany). Titrisol Buffer was purchased from Merck. Thin-layer chromatography (TLC) was performed on silica gel 60F254 plates. ^{1}H and ^{13}C NMR spectra were recorded on a Bruker (500 MHz/125 MHz and 400 MHz/100 MHz) spectrometer. Conditions are specified for each spectrum (temperature 25 °C unless specified). Chemical

shifts are reported in parts per million (ppm) relative to residual solvent and coupling constants (*J*) are reported in hertz (Hz). Signals are described as s (singlet), d (doublet), t (triplet), q (quartet), m (multiplet) and bs (broad singlet). Deuterated solvents were purchased from Euriso-top®.

Melting points were determined on a Büchi Melting Point B-540 apparatus in open capillary tubes. Semi-preparative reverse-phase high performance liquid chromatography (RP-HPLC) separations were performed on a Waters C18 SunFire™ Prep OBD™ column (5 µm, 19 mm × 150 mm) using a linear gradient (from 5% to 100% of solvent B in solvent A over 25 min; solvent A: water/0.1% trifluoroacetic acid (TFA); solvent B: acetonitrile/0.1% TFA; flow rate: 20 mL.min-1; detection at 220 nm and 254 nm). Analytical RP-HPLC was performed on final products on a Supelco C18 Ascentis® Express column (2.7 µm, 4.6 mm × 75 mm) using a linear gradient (from 5% to 100% of solvent B in solvent A over 7.5 min; solvent A: water/0.1% TFA; solvent B: acetonitrile/0.1% TFA; flow rate: 1.6 mL.min-1; detection at 220 nm and 254 nm). Purified compounds eluted as single and symmetrical peaks – thereby confirming a purity higher than 95% - at retention times *(t_R)* given below. Their molecular mass was determined by high resolution mass spectrometry (HRMS) on an Agilent Technology 6520 QTof LC/MS mass spectrometer. To establish excitation and emsission spectra, DMSO stock solutions of ligands have been diluted in water and fluorescence properties were measured on a Fluoromax 4 spectrometer (Horiba France, Les Ulis, France).

tert*-Butyl 11-(2,3-dihydroxypropylamino)-11-oxoundecylcarbamate (6).** PyBOP (828 mg, 1.59 mmol) was added to a stirred solution of 11-**(tert*-butoxycarbonylamino) undecanoic acid **5** (400 mg, 1.32 mmol) in DMF/CH_2Cl_2 (1/2, v/v, 30 mL). After 5 min at room temperature, 3-amino-1,2-propanediol (145 mg, 1.59 mmol) and *i*-Pr_2EtN (438 µL, 2.65 mmol) were added and the mixture was stirred overnight at room temperature. It was then concentrated *in vacuo*,

dissolved in EtOAc and washed sequentially with aqueous 2 M $NaHSO_4$, saturated aqueous $NaHCO_3$ and aqueous NaCl (3 times). After drying over Na_2SO_4, the solution was concentrated under reduced pressure and purified by chromatography on a silica gel column (CH_2Cl_2/MeOH, 10/0 to 9/1, v/v) to provide compound **6** as a white solid (456 mg, 92%); R_f = 0.21 (CH_2Cl_2/MeOH 96/4); mp 82-83 °C. ^{1}H NMR ($CDCl_3$, 400 MHz): δ 6.59 (bs, 1 H), 4.61 (bs, 1 H), 3.76-3.72 (m, 3 H), 4.50 (d, J = 4.9 Hz, 2 H), 3.39-3.30 (m, 2 H), 3.12-3.02 (m, 3 H), 2.18 (t, J = 7.4 Hz, 2 H), 1.81-1.78 (m, 1 H), 1.57 (m, 2 H), 1.43-1.39 (m, 2 H), 1.40 (s, 9 H), 1.22 (m, 10 H). ^{13}C NMR ($CDCl_3$, 100 MHz): δ 175.3, 156.1, 79.1, 78.8, 71.1, 63.6, 46.5, 46.4, 42.2, 40.6, 36.5, 30.0, 29.3, 29.3, 29.2, 29.7, 26.4, 26.4, 25.7. HRMS (ESI-TOF) m/z Calcd for $C_{19}H_{38}N_2O_5$ $[M+H]^+$, 374.2780; Found: 374.2773.

***tert*-Butyl 11-oxo-11-(2-oxoethylamino)undecylcarbamate (7).** $NaIO_4$ (126 mg, 0.592 mmol) was added to a solution of **6** (148 mg, 0.395 mmol) in THF/water (1/1, v/v, 7 mL). The reaction was stirred for 1 h at room temperature, concentrated *in vacuo*, dissolved in CH_2Cl_2, washed with water, and dried over Na_2SO_4. Concentration of the solution under reduced pressure gave **7** as a pale yellow oil (135 mg, 100%) which was used without further purification; R_f = 0.32 (CH_2Cl_2/MeOH 9/1, v/v). ^{1}H NMR ($CDCl_3$, 400 MHz): δ 9.60 (m, 1 H), 6.45 (bs, 1 H), 4.55 (bs, 1 H), 4.13 (m, 2 H), 3.10-3.03 (m, 3 H), 2.21 (t, J = 7.9 Hz, 2 H), 1.77 (m, 1 H), 1.58 (m, 2 H), 1.38 (s, 9 H), 1.22 (m, 12 H). ^{13}C NMR ($CDCl_3$, 100 MHz): δ 196.9, 173.6, 156.0, 78.9, 50.2, 46.3, 40.6, 36.2, 30.0, 29.4, 29.3, 29.2, 29.2, 28.4, 26.7, 26.4, 26.4, 25.5. HRMS (ESI-TOF) m/z Calcd for $C_{18}H_{34}N_2O_4$ $[M+H]^+$,, 342.2518; Found: 342.2507.

Methyl 5-(6-(*tert*-butoxycarbonylamino)hexanamido)pentanoate (10). *N*-succinimidyl 6-(*tert*-butoxycarbonylamino)hexanoate **9** (2.00 g, 6.09 mmol) and *i*-Pr_2EtN (10 mL, 60 mmol) were added to a solution of methyl 5-aminopentanoate **8** (958 mg, 7.03 mmol) in DMF (2 mL). The reaction was stirred for 4 h at room temperature, concentrated *in vacuo*, dissolved in

EtOAc, washed with aqueous NaCl (3 times). After drying over Na_2SO_4, the solution was concentrated under reduced pressure and purified by chromatography on a silica gel column (CH_2Cl_2/MeOH, 10/0 to 9/1, v/v) to provide compound **10** as a colorless oil (1.71 g, 82%); R_f = 0.34 (CH_2Cl_2/MeOH 95/5). ^{1}H NMR ($CDCl_3$, 400 MHz): δ 5.54 (bs, 1 H), 4.54 (bs, 1 H), 3.64 (s, 3 H), 3.23 (q, J = 7 Hz, 13 Hz, 2 H), 3.08 (m, 2 H), 2.32 (t, J = 7.2 Hz, 2 H), 2.13 (t, J = 7.5 Hz, 2 H), 1.65-1.60 (m, 4 H), 1.53-1.45 (m, 4 H), 1.41 (s, 9 H), 1.35-1.29 (m, 2 H). ^{13}C NMR ($CDCl_3$, 100 MHz): δ 174.1, 173.0, 156.2, 77.4, 76.3, 53.8, 51.8, 40.5, 39.1, 36.8, 33.7, 30.0, 29.2, 28.6, 26.6, 25.5, 22.3. HRMS (ESI-TOF) m/z Calcd for $C_{17}H_{32}N_2O_5$ $[M+H]^+$, 344.2311; Found: 344.2302.

5-(6-(*tert*-Butoxycarbonylamino)hexanamido)pentanoic acid (11). LiOH (1.00 g, 43.5 mmol) in water (10 mL) was added to a solution of **10** (1.50 g, 4.35 mmol) in THF (30 mL). The reaction was stirred overnight at room temperature, concentrated *in vacuo* and purified by chromatography on a silica gel column (CH_2Cl_2/MeOH 10/0 to 8/2, v/v) to provide compound **11** as a white pasty solid (1.35 g, 94%); R_f = 0.3 (CH_2Cl_2/MeOH 9/1 v/v). ^{1}H NMR (DMSO, 400 MHz): δ 7.73 (m, 1 H), 6.74 (m, 1 H), 3.00 (q, J = 7 Hz, 13 Hz, 2 H), 2.87 (q, J = 7 Hz, 13 Hz, 2 H), 2.18 (t, J = 7.1 Hz, 2 H), 2.01 (t, J = 7.3 Hz, 2 H), 1.47-1.34 (m, 17 H), 1.23-1.16 (m, 2 H). HRMS (ESI-TOF) m/z Calcd for $C_{16}H_{30}N_2O_5$ $[M+H]^+$, 330.2156; Found: 330.2148.

***tert*-Butyl 6-(5-(2,3-dihydroxypropylamino)-5-oxopentylamino)-6-oxohexylcarbamate (12).** PYBOP (756 mg, 1.45 mmol) was added to a stirred solution of **11** (400 mg, 1.21 mmol) in DMF (10 mL). After 5 min at room temperature, 3-amino-1,2-propanediol (132 mg, 1.45 mmol) and *i*-Pr_2EtN (782 µL, 4.84 mmol) were added and the mixture was allowed to react overnight at room temperature. It was then concentrated *in vacuo* and purified by chromatography over a silica gel column (CH_2Cl_2/MeOH, from 10/0 to 8/2, v/v) to provide compound **12** as a white pasty solid (458 mg, 94%); R_f = 0.32 (CH_2Cl_2/MeOH 9/1). ^{1}H NMR ($CDCl_3$, 400 MHz): δ 7.95 (bs, 1H), 3.73-3.67 (m, 1H), 3.54-3.46 (m, 2H), 3.38-3.41 (m, 1H),

3.22-3.16 (m, 4H), 3.2 (t, J = 7.0 Hz, 2H), 2.24 (t, J = 7.4 Hz, 2H), 2.18 (t, J = 2.18 Hz, 2H), 1.66-1.57 (m, 4H), 1.55-1.45 (m, 4H), 1.43 (s, 12H), 1.36-1.28 (m, 2H). ^{13}C NMR ($CDCl_3$, 100 MHz): δ 176.6, 176.2, 158.6, 79.9, 72.2, 65.1, 43.5 (2C), 41.3, 40.0 (2C), 37.1, 36.6, 30.8, 30.0, 28.9, 27.6, 26.9, 24.3. HRMS (ESI-TOF) m/z Calcd for $C_{19}H_{37}N_3O_6$ $[M+H]^+$, 403.2682; Found: 403.2686.

***tert*-Butyl 6-oxo-6-(5-oxo-5-(2-oxoethylamino)pentylamino)hexylcarbamate (13).** $NaIO_4$ (159 mg, 0.743 mmol) was added to a stirred solution of **12** (200 mg, 0.495 mmol) in a mixture of THF/water (1/1, v/v, 7 mL). The reaction was stirred for 1 h at room temperature, concentrated *in vacuo*, dissolved in CH_2Cl_2, washed with water and dried over Na_2SO_4. Concentration of the solution under reduced pressure gave **13** as a yellow oil (184 mg, 100%) which was used without further purification R_f = 0.4 (CH_2Cl_2/MeOH 9/1, v/v). ^{1}H NMR ($CDCl_3$, 400 MHz): δ 7.95 (m, 1H), 4.55 (t, J = 5.3 Hz, 1H), 3.24-3.15 (m, 6H), 3.02 (t, J = 7.0 Hz, 2H), 2.22 (t, J = 7.5 Hz, 2H), 2.18 (t, J = 7.5 Hz, 2H), 1.65-1.57 (m, 4H), 1.54-1.40 (m, 4H), 1.43 (s, 9H), 1.36-1.29 (m, 2H). ^{13}C NMR ($CDCl_3$, 100 MHz): δ 176.2, 176.1, 176.0, 158.5, 97.3, 79.8, 55.0, 45.5, 41.2, 39.9, 37.1, 36.4, 30.7, 29.8, 28.9, 27.5, 26.7, 24.2. HRMS (ESI-TOF) m/z Calcd for $C_{18}H_{33}N_3O_5$ $[M+H]^+$, 371.2420; Found: 371.2418.

***trans*-5-Chloro-2,3,3a,12b-tetrahydro-1*H*-dibenzo[2,3:6,7]oxepino[4,5-*c*]pyrrole (17) (Norasenapine).** Asenapinium maleate **14** (50 mg, 0.124 mmol) in CH_2Cl_2/MeOH (1/2, v/v, 2.8 mL) was added to PL-TBD resin (0.37 mmol) preswollen in CH_2Cl_2/MeOH (1/1, v/v, 9 mL). The reaction was shaken overnight at room temperature and filtered. The filtrate was concentrated *in vacuo*. The free base (35.56 mg, 0.124 mmol) was dissolved in 1,2-dichloroethane (2 mL) and allowed to react overnight with 1-chloroethyl chloroformate (134 µL, 1.24 mmol) at 100 °C. The mixture was then concentrated *in vacuo*, dissolved in methanol, stirred for 2 h at reflux, concentrated *in vacuo*, and purified by chromatography over a silica gel column (CH_2Cl_2/MeOH 10/0 to 9/1, v/v) to provide compound **17** as a white

solid (26.8 mg, 79%); R_f = 0.25 (CH_2Cl_2/MeOH 9/1, v/v); mp 236 °C. ^{1}H NMR (DMSO, 400 MHz): δ 7.27-7.08 (m, 7 H), 3.47-3.42 (m, 2 H), 3.31-3.23 (m, 2 H), 3.10-3.03 (m, 2 H). ^{13}C NMR (DMSO, 100 MHz): δ 154.8, 153.9, 134.3, 131.8, 128.1, 127.6, 127.5, 127.3, 127.2, 124.5, 122.5, 120.6, 50.2, 50.1, 45.1, 44.7. RP-HPLC purity: > 95%, t_R = 3.66. HRMS (ESI-TOF) m/z Calcd for $C_{16}H_{14}ClNO$ $[M+H]^+$, 271.0764; Found: 271.0763.

***tert*-Butyl 2-(*trans*-5-chloro-1,3,3a,12b-tetrahydro-2*H*-dibenzo[2,3:6,7]oxepino[4,5-*c*]pyrrol-2-yl)ethylcarbamate (18).** *N*-Boc-2-aminoacetaldehyde (7 mg, 0.044 mmol), NEt_3 (4 μL, 0.029 mmol) and $NaBH_3CN$ (2.78 mg, 0.044 mmol) were added to a solution of compound **17** trifluoroacetate (11.4 mg, 0.029 mmol) in methanol (0.34 mL). The reaction was stirred for 2 h at 25 °C, concentrated *in vacuo*, and purified by chromatography over a silica gel column (CH_2Cl_2/MeOH 100/0 to 99/1, v/v) to provide compound **18** as a colorless oil (11.8 mg, 96%); R_f = 0.05 (CH_2Cl_2 100%). ^{1}H NMR (DMSO, 500 MHz): δ 7.27-7.10 (m, 7 H), 6.76 (t, J = 5.1 Hz, 1 H), 3.45-3.40 (m, 2 H), 3.23 (m, 2 H), 3.12 (m, 2 H), 3.04 (m, 2 H), 2.75-2.70 (m, 1 H), 2.62-2.58 (m, 1 H), 1.38 (s, 9 H). ^{13}C NMR (DMSO, 125 MHz): δ 155.6, 154.7, 153.8, 134.3, 131.8, 128.0, 127.7, 127.4, 127.2, 126.9, 124.4, 122.5, 120.6, 77.5, 56.7, 56.4, 55.7, 43.8, 43.5, 43.4, 43.1, 42.6, 28.2. RP-HPLC purity: > 95%, t_R = 4.52. HRMS (ESI-TOF) m/z Calcd for $C_{23}H_{27}ClN_2O_3$ $[M+H]^+$, 414.1710; Found 414.1720.

2-(*trans*-5-Chloro-1,3,3a,12b-tetrahydro-2*H*-dibenzo[2,3:6,7]oxepino[4,5-*c*]pyrrol-2-yl)ethan-1-amine, trifluoroacetate (19). TFA (0.5 mL) was added to a solution of **18** (10.5 mg, 0.025 mmol) in CH_2Cl_2 (2 mL). The reaction was stirred for 2 h at room temperature, concentrated *in vacuo* and purified by RP-HPLC to provide compound **19** (trifluoroacetate) as a yellow solid (7 mg, 65%); R_f = 0.27 (CH_2Cl_2/NH_4OH in MeOH 7 M, 95/5); mp 108 °C. ^{1}H NMR (DMSO, 400 MHz): δ 8.25 (bs, 2H), 7.39-7.18 (m, 7H), 4.10-3.57 (m, 8H), 3.32-3.29 (m, 2H). ^{13}C NMR (DMSO, 100 MHz): δ 154.6, 153.8, 130.9, 128.7, 128.5, 128.4, 127.5,

127.3, 124.8, 122.9, 121.0, 56.4, 56.1, 52.4, 42.0, 41.7, 34.9. RP-HPLC purity: > 95%, t_R = 3.28. HRMS (ESI-TOF) m/z Calcd for $C_{18}H_{19}ClN_2O$ $[M+H]^+$, 314.1186; Found 314.1185.

***tert*-Butyl 11-(2-(*trans*-5-chloro-1,3,3a,12b-tetrahydro-2*H*-dibenzo[2,3:6,7]oxepino[4,5-*c*]pyrrol-2-yl)ethylamino)-11-oxoundecylcarbamate (20).** Aldehyde **7** (20 mg, 0.059 mmol) and $NaBH_3CN$ (2.8 mg, 0.044 mmol) were added to a solution of **17** (8 mg, 0.03 mmol) in MeOH (1 mL). The reaction was stirred 2 h at room temperature, concentrated *in vacuo* and purified by chromatography over a silica gel column (CH_2Cl_2/MeOH 100/0 to 95/5, v/v) to provide compound **20** as a yellow oil (14 mg, 79%); R_f = 0.4 (CH_2Cl_2/MeOH 97.5/2.5). ^{1}H NMR (DMSO, 500 MHz): δ 7.80 (m, 1H), 7.30-7.11 (m, 7H), 6.74 (m, 1H), 3.51-3.47 (m, 2H), 3.31-3.25 (m, 4H), 3.11 (m, 2H), 2.87 (m, 2H), 2.78 (m, 1H), 2.68 (m, 1H), 2.07 (t, J = 7.2 Hz, 2H), 1.49 (m, 2H), 1.37 (s, 9H), 1.33 (m, 2H), 1.23-1.19 (m, 12H). ^{13}C NMR (DMSO, 125 MHz) δ 172.1, 155.5, 154.7, 153.8, 134.2, 131.7, 128.0, 127.8, 127.4, 127.2, 126.9, 124.4, 122.5, 120.6, 77.2, 56.7, 56.5, 55.4, 43.7, 43.4, 37.6, 36.6, 35.4, 29.4, 29.0, 28.9, 28.8, 28.7, 28.6, 28.2, 26.2, 25.3. RP-HPLC purity: > 95%, t_R = 5.61. HRMS (ESI-TOF) m/z Calcd for $C_{34}H_{48}ClN_3O_4$ $[M+H]^+$, 597.3333; Found: 597.3338.

11-((2-(*trans*-5-Chloro-1,3,3a,12b-tetrahydro-2*H*-dibenzo[2,3:6,7]oxepino[4,5-*c*]pyrrol-2-yl)ethyl)amino)-11-oxoundecan-1-amine (21). TFA (0.5 mL) was added to a solution of **20** (14 mg, 0.023 mmol) in CH_2Cl_2 (2 mL). The reaction was stirred for 2 h at room temperature, concentrated *in vacuo* and purified by RP-HPLC to provide compound **21** as a yellow oil (free base: 11.5 mg, 98%); R_f = 0.27 (CH_2Cl_2/ NH_3-MeOH 7 M 95/5). R_f = 0.44 (CH_2Cl_2/ NH_3-MeOH 7 M, 9/1). ^{1}H NMR (DMSO, 400 MHz): δ 7.79 (t, J = 5.8 Hz, 1 H), 7.28-7.09 (m, 7 H), 3.51-3.43 (m, 2 H), 3.26-3.12 (m, 6 H), 3.08-3.03 (m, 2 H), 2.77-2.71 (m, 1 H), 2.65-2.59 (m, 1 H), 2.07 (t, J = 7.3 Hz, 2 H) 1.52-1.45 (m, 2 H), 1.32-1.19 (m, 16 H). ^{13}C NMR (DMSO, 100 MHz): δ 172.0, 154.7, 153.8, 134.3, 131.8, 128.0, 127.6, 127.3, 127.1, 126.9, 124.3, 122.4, 120.5, 56.7, 56.5, 55.4, 43.8, 43.5, 41.3, 37.8, 35.4, 32.7, 29.0, 28.9, 28.8, 28.6,

26.3, 25.3. RP-HPLC purity: > 95%, t_R = 3.85. HRMS (ESI-TOF) m/z Calcd for $C_{29}H_{40}ClN_3O_2$ $[M+H]^+$, 497.2809; Found: 497.2808.

***trans*-2-(14-Azido-3,6,9,12-tetraoxatetradecyl)-5-chloro-2,3,3a,12b-tetrahydro-1*H*-dibenzo[2,3:6,7]oxepino[4,5-*c*]pyrrole (22).** Compound **4** (7.5 mg, 0.022 mmol) and K_2CO_3 (6 mg, 0.045 mmol) were added to a solution of compound **17** (7 mg, 0.018 mmol) in acetonitrile (200 μL). The reaction was stirred overnight at room temperature, sonicated for 2 h at 50 °C, filtered, and purified by column chromatography to provide compound **22** (4.3 mg, 47%). ^{1}H NMR ($CDCl_3$, 400 MHz): δ 7.31-7.06 (m, 7 H), 4.47-4.14 (m, 2 H), 3.98-3.80 (m, 6H), 3.71-3.46 (m, 16 H), 3.35 (t, J = 5.1 Hz, 2H). ^{13}C NMR ($CDCl_3$, 100 MHz): δ 155.4, 154.3, 129.3, 128.2, 127.9, 127.0, 126.9, 124.5, 122.6, 121.2, 70.7, 70.6, 70.6, 70.0, 57.6, 57.4, 56.1, 50.7, 43.6, 43.5. HRMS (ESI-TOF) m/z Calcd for $C_{26}H_{33}ClN_4O_5$ $[M+H]^+$, 516.2135; Found: 516.2139.

14-(*trans*-5-Chloro-1,3,3a,12b-tetrahydro-2*H*-dibenzo[2,3:6,7]oxepino[4,5-*c*]pyrrol-2-yl)-3,6,9,12-tetraoxatetradecan-1-amine (23). Compound **22** (25 mg, 0.046 mmol) in THF (1.5 mL) and water (0.4 mL) were added to PS-PPh3 resin (46 mmol) preswollen in THF (1.5 mL) for 25 min. The reaction was shaken overnight at room temperature and filtered. The filtrate was concentrated *in vacuo* and purified by RP-HPLC to provide compound **23** (free base; 21.5 mg, 95%). ^{1}H NMR (MeOD, 400 MHz): δ 8.82-8.68 (m, 7H), 5.78-5.57 (m, 4 H), 5.42-5.39 (m, 6 H), 5.24-5.22 (m, 2 H), 5.20-5.17 (m, 3 H), 5.16-5.13 (m, 4 H), 5.11-5.07 (m, 4 H), 5.05-5.02 (m, 3 H), 4.99-4.95 (m, 2 H). ^{13}C NMR (MeOD, 100 MHz): δ 156.1 131.0, 130.3, 130.1, 129.0, 127.7, 126.3, 124.3, 122.6, 73.8, 71.6, 71.5, 66.4, 62.3, 58.5, 58.2, 57.5, 44.0, 43.5. RP-HPLC purity: > 95%, t_R = 3.42. HRMS (ESI-TOF) m/z Calcd for $C_{26}H_{35}ClN_2O_5$ $[M+H]^+$, 490.2234; Found: 490.2231.

***tert*-Butyl 6-(5-(2-(*trans*-5-chloro-1,3,3a,12b-tetrahydro-2*H*-dibenzo[2,3:6,7]oxepino[4,5-*c*]pyrrol-2-yl)ethylamino)-5-oxopentylamino)-6-oxohexylcarbamate (24).** Aldehyde **13**

(20.5 mg, 0.055 mmol) and $NaBH_3CN$ (3.4 mg, 0.055 mmol) were added to a solution of **17** (10 mg, 0.037 mmol) in MeOH (1 mL). The reaction was stirred 2 h at room temperature, concentrated *in vacuo* and purified by chromatography over a silica gel column (CH_2Cl_2/MeOH 100/0 to 80/20, v/v) to provide compound **24** as a yellow oil (14.8 mg, 64%); R_f = 0.25 (CH_2Cl_2/MeOH 95/5 v/v). ^{1}H NMR (DMSO, 500 MHz): δ 7.79 (t, J = 5.8 Hz, 1 H), 7.71 (t, J = 5.6 Hz, 1 H), 7.27-7.09 (m, 7 H), 6.74 (t, J = 5.4 Hz, 1 H), 3.51-3.41 (m, 2 H), 3.25-3.21 (m, 4 H), 3.05 (m, 2 H), 3.01 (m, 2 H), 2.87 (m, 2 H), 2.76-2.71 (m, 1 H), 2.64-2.59 (m, 1 H), 2.08 (m, 2 H), 2.01 (m, 2 H), 1.52-1.41 (m, 4 H), 1.39-1.30 (m, 4 H), 1.35 (s, 9 H), 1.21-1.14 (m, 2 H). ^{13}C NMR (DMSO, 125 MHz) δ 171.9, 171.8, 155.5, 154.7, 153.8, 134.3, 131.9, 128.0, 127.7, 127.4, 127.2, 126.9, 124.4, 122.5, 120.6, 77.2, 56.7, 56.6, 55.6, 54.8, 43.8, 43.5, 38.1, 37.8, 35.4, 35.0, 29.3, 28.8, 28.2, 26.0, 25.0, 22.8. HRMS (ESI-TOF) m/z Calcd for $C_{34}H_{47}ClN_4O_5$ $[M+H]^+$, 626.3235; Found: 626.3247.

6-((5-((2-(*trans*-5-Chloro-1,3,3a,12b-tetrahydro-2*H*-dibenzo[2,3:6,7]oxepino[4,5-*c*]pyrrol-2-yl)ethyl)amino)-5-oxopentyl)amino)-6-oxohexan-1-amine, trifluoroacetate (25). TFA (0.5 mL) was added to a solution of **24** (14 mg, 0.022 mmol) in CH_2Cl_2 (2 mL). The reaction was stirred for 2 h at room temperature, concentrated *in vacuo* and purified by RP-HPLC to provide compound **25** as a colorless oil (10.5 mg, 89%); R_f = 0.42 (CH_2Cl_2/MeOH-NH_4 7M 85/15 v/v). ^{1}H NMR (DMSO, 400 MHz): δ 7.82 (t, J = 6.0 Hz, 1H), 7.76 (t, J = 5.3 Hz, 1H), 7.28-7.11 (m, 7H), 5.1-4.2 (bs, 2H), 3.47-3.44 (m, 2H), 3.26-3.21 (m, 4H), 3.07-2.99 (m, 4H), 2.74-2.60 (m, 4H), 2.08 (m, 2H), 2.03 (m, 2H), 1.50-1.43 (m, 6H), 1.38-1.35 (m, 2H), 1.26-1.22 (m, 2H). ^{13}C NMR (DMSO, 100 MHz): δ 171.9, 171.7, 154.7, 153.8, 134.3, 131.9, 129.1, 128.0, 127.7, 127.4, 127.2, 127.0, 124.4, 122.5, 120.6, 56.7, 56.6, 55.6, 43.8, 43.5, 38.1, 37.8, 35.2, 35.0, 28.9, 28.8, 25.7, 24.9, 22.8. RP-HPLC purity: > 95%, t_R = 3.41. HRMS (ESI-TOF) m/z Calcd for $C_{29}H_{39}ClN_4O_3$ $[M+H]^+$, 526.2711; Found: 526.2707.

General procedure for the labeling of amines 19, 21, 23, and 25 with fluorophores. i-Pr_2EtN (10 equiv) and 6-(fluorescein-5-carboxamido) hexanoic acid, succinimidyl ester (5-SFX-NHS), 2-((1E,3E)-5-((Z)-3-(4-((2,5-dioxopyrrolidin-1-yl)oxy)-4-oxobutyl)-1-ethyl-3-methyl-5-sulfoindolin-2-ylidene)penta-1,3-dien-1-yl)-1-ethyl-3,3-dimethyl-3H-indol-1-ium-5-sulfonate (DY647-NHS) or Fluorescein-5-N-hydroxysuccinimide ester (FLUO-NHS) (1 equiv) were added to a solution of amine (1 equiv) in DMSO. The reaction was stirred for 2 h at room temperature and was monitored by analytical RP-HPLC. The products were purified by RP-HPLC.

5-(6-(2-(*trans*-5-Chloro-1,3,3a,12b-tetrahydro-2*H*-dibenzo[2,3:6,7]oxepino[4,5-*c*]pyrrol-2-yl)ethylamino)-6-oxohexylcarbamoyl)-2-(6-hydroxy-3-oxo-3*H*-xanthen-9-yl)benzoic acid (26). Yellow powder. RP-HPLC purity: > 95%, t_R = 4.38. UV (titrisol buffer): λ_{max} excitation 501 nm, λ_{max} emission 527 nm. HRMS (ESI-TOF) m/z Calcd for $C_{45}H_{40}ClN_3O$ $[M+H]^+$, 785.2504; Found: 785.2484.

2-((1E,3E,5E)-5-(3-(4-((2-(5-Chloro-3,3a-dihydro-1H-dibenzo[2,3:6,7]oxepino[4,5-c]pyrrol-2(12bH)-yl)ethyl)amino)-4-oxobutyl)-1-ethyl-3-methyl-5-sulfoindolin-2-ylidene)penta-1,3-dien-1-yl)-1-ethyl-3,3-dimethyl-5-sulfo-3H-indol-1-ium (27). Blue powder. RP-HPLC purity: > 95%, t_R = 3.87. UV (titrisol buffer): λ_{max} excitation 651 nm, λ_{max} emission 665 nm. HRMS (ESI-TOF) m/z Calcd for $C_{50}H_{55}ClN_4O_8S_2$ $[M+H]^+$, 938.3149; Found: 938.3150.

5-((6-((11-((2-(5-Chloro-3,3a-dihydro-1H-dibenzo[2,3:6,7]oxepino[4,5-c]pyrrol-2(12bH)-yl)ethyl)amino)-11-oxoundecyl)amino)-6-oxohexyl)carbamoyl)-2-(6-hydroxy-3-oxo-3H-xanthen-9-yl)benzoic acid (28). Yellow powder. RP-HPLC purity: > 95%, t_R = 4.80. UV (titrisol buffer): λ_{max} excitation 494 nm, λ_{max} emission 525 nm. HRMS (ESI-TOF) m/z Calcd for $C_{56}H_{61}ClN_4O_9$ $[M+H]^+$, 968.4127; Found: 968.4122.

2-((1E,3E,5E)-5-(3-(4-((11-((2-(5-Chloro-3,3a-dihydro-1H-dibenzo[2,3:6,7]oxepino[4,5-c]pyrrol-2(12bH)-yl)ethyl)amino)-11-oxoundecyl)amino)-4-oxobutyl)-1-ethyl-3-methyl-5-sulfoindolin-2-ylidene)penta-1,3-dien-1-yl)-1-ethyl-3,3-dimethyl-5-sulfo-3H-indol-1-ium (29). Blue powder. RP-HPLC purity: > 95%, t_R = 4.36. UV (titrisol buffer): λ_{max} excitation 653 nm, λ_{max} emision 665 nm. HRMS (ESI-TOF) m/z Calcd for $C_{61}H_{77}ClN_5O_9S_2$ $[M+H]^+$, 1121.4773; Found: 1121.4757.

5-((1-(5-Chloro-3,3a-dihydro-1H-dibenzo[2,3:6,7]oxepino[4,5-c]pyrrol-2(12bH)-yl)-16-oxo-3,6,9,12-tetraoxa-15-azahenicosan-21-yl)carbamoyl)-2-(6-hydroxy-3-oxo-3H-xanthen-9-yl)benzoic acid (30). Yellow powder. RP-HPLC purity: > 95%, t_R = 4.35. UV (titrisol buffer): λ_{max} excitation 502 nm, λ_{max} emission 522 nm. HRMS (ESI-TOF) m/z Calcd for $C_{53}H_{56}ClN_3O_{12}$ $[M+H]^+$, 961.3553; Found: 961.3535.

2-((1E,3E,5E)-5-(3-(1-(5-Chloro-3,3a-dihydro-1H-dibenzo[2,3:6,7]oxepino[4,5-c]pyrrol-2(12bH)-yl)-16-oxo-3,6,9,12-tetraoxa-15-azanonadecan-19-yl)-1-ethyl-3-methyl-5-sulfoindolin-2-ylidene)penta-1,3-dien-1-yl)-1-ethyl-3,3-dimethyl-5-sulfo-3H-indol-1-ium (31). Blue powder. RP-HPLC purity: > 95%, t_R = 3.92. UV (titrisol buffer): λ_{max} excitation 660 nm, λ_{max} em$_{ission}$ 668 nm. HRMS (ESI-TOF) m/z Calcd for $C_{58}H_{71}ClN_4O_{12}S_2$ $[M+H]^+$, 1114.4198; Found: 1114.4199.

5-((6-((6-((5-((2-(5-Chloro-3,3a-dihydro-1H-dibenzo[2,3:6,7]oxepino[4,5-c]pyrrol2(12bH)yl)ethyl)amino)-5-oxopentyl)amino)-6-oxohexyl)amino)-6-oxohexyl)carbamoyl)-2-(6-hydroxy-3-oxo-3H-xanthen-9-yl)benzoic acid (32). Yellow powder (143 nmol, 14%). RP-HPLC purity: >95%, t_R = 4.22. UV (titrisol buffer): λ_{max} excitation 500 nm, λ_{max} emission 526 nm. HRMS (ESI-TOF) m/z Calcd for $C_{56}H_{60}ClN_5O_{10}$ $[M+H]^+$, 997.4028; Found: 997.4010.

2-((1E,3E,5E)-5-(3-(4-((6-((5-((2-(5-Chloro-3,3a-dihydro-1H-dibenzo[2,3:6,7]oxepino[4,5-c]pyrrol-2(12bH)-yl)ethyl)amino)-5-oxopentyl)amino)-6-

oxohexyl)amino)-4-oxobutyl)-1-ethyl-3-methyl-5-sulfoindolin-2-ylidene)penta-1,3-dien-1-yl)-1-ethyl-3,3-dimethyl-3H-indol-1-ium-5-sulfonate (33). Blue powder (304 nmol, 30%). UV (MeOH): λ_{max} 649 nm. RP-HPLC purity: > 95%, t_R = 3.90. UV (titrisol buffer): λ_{max} excitation 655 nm, λ_{max} emission 665 nm. HRMS (ESI-TOF) m/z Calcd for $C_{61}H_{77}ClN_6O_{10}S_2$ $[M+2H]^{++}$:1152.4831; Found: 1152.4799.

5-((6-((6-((5-((2-(5-Chloro-3,3a-dihydro-1H-dibenzo[2,3:6,7]oxepino[4,5-c]pyrrol-2(12bH)-yl)ethyl)amino)-5-oxopentyl)amino)-6-oxohexyl)amino)-6-oxohexyl)carbamoyl)-2-(6-hydroxy-3-oxo-3H-xanthen-9-yl)benzoic acid (34). Yellow powder (340 nmol, 34%). UV (titrisol buffer): λ_{max} 498 nm. RP-HPLC purity: >95%, t_R = 4.27. UV (titrisol buffer): λ_{max} excitation 495 nm, λ_{max} emission 524 nm. HRMS (ESI-TOF) m/z Calcd for $C_{50}H_{49}ClN_4O_9$ $[M+H]^+$, 884.3188; Found: 884.3153.

Biology

Reagents. The 96-well plates (ref. 655086) were purchased from Greiner Bio-One (www.gbo.com). Substrate SNAP-Lumi4-Tb (ref. SSNPTBX) was provided by Cisbio Bioassays. All unlabelled ligands were purchased from Tocris (Bristol, UK). All SNAP-GPCR plasmids were provided by Cisbio Bioassays. Details can be found at http://www.cisbio.com/drug-discovery/receptor-binding-assays. Tag-lite labeling medium (ref. LABMED) was provided by Cisbio Bioassays.

Cell Culture. HEK293T wild-type cells were maintained in Dulbecco's modified Eagle's medium (DMEM) glutaMAX (1966-021; Invitrogen, Carlsbad, CA) supplemented with 10% fetal calf serum.

Transfection procedures. Reverse transient transfections were performed on adherent HEK293T cells in 96-well plates using cell density from 50,000 to 100,000 cells per well. Prior to cell plating, wells were precoated with 50 µL poly-L-ornithine for 30 min at 37°C. For all assays, transfection mixes were prepared using 100 ng of the GPCR plasmid, 0.5 µL of

Lipofectamine 2000 (Life Technologies) and 50 µl of OptiMEM, Glutamax™ culture medium per well. Before addition in plates, transfection mixes were preincubated 20 min at room temperature. Then, 100 µL of HEK293T cells were added in each well. Plates were incubated at 37 °C under 5% CO_2 for 24 h before ligand binding assays.

Time-resolved FRET Binding Assays. At first, HEK293T cells transiently expressing SNAP-tagged GPCRs were treated with substrate SNAP-Lumi4-Tb. Cell culture medium was removed from the 96-well plates and 100 nM of reagent, previously diluted in Tag-lite labeling medium, was added (100 µL per well) before an incubation of 1 h at 37 °C under 5% CO_2. The excess of reagent was removed by 4 washes with 100 µL of Tag-lite labeling medium. Ligand Binding experiments were then performed with plates containing 50 µL of Tag-lite labeling medium. 25 µL of unlabeled compound or Tag-lite labeling medium was added, followed by the addition of 25 µL of fluorescent ligand. Plates were then incubated at RT in the dark for 1 hour before signal detection. Binding affinities were determined by incubating the cells at RT with increasing concentrations of the desired fluorescent ligand. For each fluorescent ligand concentration, the nonspecific binding was determined in presence of an excess of unlabeled compound (10 µM asenapine). Both fluorescent ligands and unlabeled compounds were diluted in the Tag-lite labeling medium. For competition binding assays, fluorescent ligands at fixed concentration (**30** and **31** were respectively used at 70 nM and 10 nM on $5HT_{2b}$ receptor; **29** and **31** were respectively used at 4 nM and 17 nM on $5HT_{1d}$ receptor; **26** was used at 10 nM on $5HT_{2c}$, $5HT_6$ and D_4 receptors; **31** was used at 78 nM on D_4 receptor), are used in presence of increasing concentrations of unlabeled ligands. Plates are incubated 1 hour in the dark at room temperature before signal detection.

Data Analysis. Signal detection was performed on Infinite F500 (Tecan, Männedorf, Switzerland). Time-resolved FRET readouts were recorded and analyzed as described previously (Zwier, J. M. J. Biomol. Screening 2010, 15, (10), 1248-1259). All binding data

were analyzed with GraphPad Prism (GraphPad Software, Inc., San Diego, CA). K_d values of the fluorescent ligands were obtained from saturation curves of the specific binding. Specific binding was determined by subtracting the nonspecific homogenous time-resolved fluorescence (HTRF) ratio from the total HTRF ratio. Competition data were analyzed according to a sigmoid model by nonlinear regression. Ki values of unlabeled compounds were calculated from IC_{50} of binding competition experiments according to the Cheng and Prusoff equation: $Ki = IC_{50}/ (1+([L]/K_d))$, where IC_{50} is the concentration of unlabeled analogue leading to half-maximal inhibition of specific binding, K_d is its affinity for the receptor studied, and [L] is the concentration of the fluorescent probe present in the assay. All results are expressed as the Mean ± SEM of at least three independent experiments.

ANCILLARY INFORMATION

a. **Supporting information Availability:** Molecular formula strings; Fluorescence properties of ligands **26-34.**

b. **Corresponding Author Information:** mhibert@unistra.fr

c. **Author Contributions:** # C.H. and C.B. contributed equally to the work.

d. **Abbreviation used:** ^{13}C NMR carbon 13 nuclear magnetic resonance; DMEM Dulbecco's modified Eagle's medium; DMF dimethylformamide; DMSO dimethylsulfoxyde; DY647 3-[5-[(2E)-1,3-dimethyl-2-[(2E,4E)-5-(1,3,3-trimethylindol-1-ium-2-yl)penta-2,4-dienylidene]indol-3-yl]pentoxy-[di(propan-2-yl)amino]phosphanyl]oxypropanenitrile;4-methylbenzenesulfonate; EL extracellular loops; ESI-TOF electron spray ionization-time of flight; GPCRs G protein coupled receptors; FDA, Food and Drug Administration; FRET fluorescence resonance energy transfer; HTS high throughput screening; FLUO Fluorescein; 1H NMR proton nuclear

magnetic resonance; HRMS high resolution mass spectrometry; *J* coupling constant; PL-TBD, polymer linked 1,5,7-triazabicyclo[4.4.0]dec-5-ene; ppm parts per million; PS-PPh_3 diphenylphosphino-polystyrene; PyBoP benzotriazol-1-yl-oxytripyrrolidinophosphonium hexafluorophosphate; R_f retention factor; RP-HPLC reverse-phase high performance liquid chromatography; SEM standard error of the mean; 5-SFX 6-(fluorescein-5-carboxamido) hexanoic acid, succinimidyl ester; S/N signal to noise; TFA Trifluoroacetic Acid; THF tetrahydrofuran; TLC thin layer chromatography; TM transmembrane domain; t_R HPLC retention time; TR-FRET time-resolved FRET.

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Table 1. Determination of the Binding Affinities of Fluorescent Probes for Serotonin Receptors Using TR-FRET Binding Assays.[a]

Cpd	Ligand Binding TR-FRET assay																							
	Serotonin receptors																							
	5-HT$_{1a}$		5-HT$_{1b}$		5-HT$_{1d}$		5-HT$_{1e}$		5-HT$_{1f}$		5-HT$_{2a}$		5-HT$_{2b}$		5-HT$_{2c}$		5-HT$_4$		5-HT$_{5a}$		5-HT$_6$		5-HT$_7$	
Id *Code*[e]	K_d[b]	S/N[c]	K_d	S/N	K_d	S/N	K_d	S/N	K_d	S/N	K_d	S/N	K_d	S/N	K_d	S/N	K_d	S/N	K_d	S/N	K_d	S/N	K_d	S/N
26 SL-S	>1000	n[d]	169±67	5	>1000	n	>1000	n	656±157	2	>1000	n	>1000	n	20±4	25	>1000	n	>1000	n	49±28	13	>1000	**n**
27 SL-D	>1000	n	>1000	n	>1000	n	>1000	n	>1000	n	>1000	n	>1000	n	>1000	n	>1000	n	>1000	n	>1000	n	>1000	**n**
28 LL-S	>1000	n	>1000	2	45±6	2	>1000	n	>1000	n	>1000	n	>1000	n	174±36	7	>1000	n	>1000	n	999±349	5	>1000	**n**
29 LL-D	>1000	n	63±37	2	50±3	5	>1000	n	>1000	n	>1000	n	40±6	3	36±21	2.5	>1000	n	>1000	n	122±55	7	47±4	**3**
30 PL-S	31±7	6	>1000	n	50±15	2	>1000	n	>1000	n	88±10	4	27±16	2.5	134±24	37	>1000	n	>1000	n	>1000	7	666±309	**2**
31 PL-D	32±8	9.5	>1000	3.5	17±3	9.5	>1000	n	>1000	n	17±7	2	8±3	6	53±16	65	>1000	n	>1000	n	>1000	5	36±9	**2**
32 VLL-S	>1000	n	121±45	2	>1000	n	>1000	n	>1000	n	>1000	n	>1000	n	119±44	5.5	>1000	n	>1000	n	>1000	n	>1000	**n**
33 VLL-D	3±2	2	106±40	2	53±20	2	>1000	n	>1000	n	>1000	n	>1000	n	93±44	3	>1000	n	>1000	n	55±35	2	>1000	**n**

34	VLL-F	>1000	n	>1000	2	>1000	2	>1000	n	>1000	n	>1000	n	>1000	n	104±38	3.5	>1000	n	>1000	n	>1000	n	>1000	**n**

[a]Values represent the Mean±SD from at least three independent experiments. [b]K_d values in nM units. [c]S/N represents the signal-to-noise ratio calculated in the binding assay as the ratio Total Signal/Nonspecific Signal at the K_d value. S/N was calculated at 1000 nM for K_d values over 1000 nM. [d]n indicates S/N < 2. [e]SL-S = small linker-SFX; SL-D = small linker-DY647; LL-S =long linker-SFX; LL-D = long linker-DY647; PL-S = pegylated linker-SFX; PL-D = pegylated linker-DY647; VLL-S = very long linker-SFX; VLL-D = very long linker-DY647; VLL-F = very long linker-Fluorescein.

Table 2. Determination of the Binding Affinities of Fluorescent Probes for Dopamine and Histamine Receptors Using TR-FRET Binding Assays.[a]

Cpd		Ligand Binding TR-FRET assay																	
		Dopamine receptors										Histamine receptors							
		D_1		D_2		D_3		D_4		D_5		H_1		H_2		H_3		H_4	
Id	*code*[e]	K_d[b]	S/N[c]	K_d	S/N	K_d	S/N	K_d	S/N	K_d	S/N	K_d	S/N	K_d	S/N	K_d	S/N	K_d	S/N
26	SL-S	60±3	11	6±1	4.5	20±3	6	40±4	10	>1000	n[d]	42±3	15	395±23	5	>1000	n	>1000	n
27	SL-D	>1000	n	>1000	n	>1000	n	>1000	n	>1000	n	105±21	3	>1000	n	>1000	n	>1000	n
28	LL-S	>1000	3.5	146±35	4	255±60	6	>1000	n	>1000	n	122±17	23	>1000	n	>1000	n	>1000	n
29	LL-D	892±383	2	76±23	11.5	201±58	6	204±51	2.5	>1000	n	104±19	14	>1000	n	>1000	n	>1000	n
30	PL-S	>1000	3.5	46±6	62	73±19	32	560±184	11	>1000	n	82±18	32	>1000	n	>1000	n	>1000	n
31	PL-D	>1000	5	13±2	27	24±5	125	56±12	11	>1000	n	36±6	29	>1000	2	>1000	n	>1000	n
32	VLL-S	>1000	n	65±29	4	>1000	2	>1000	n	>1000	n	316±114	12	310±122	3	>1000	n	>1000	n
33	VLL-D	>1000	n	42±13	4.5	15±5	3	>1000	n	>1000	n	20±7.5	8	>1000	n	>1000	n	>1000	n
34	VLL-F	>1000	n	105±62	4	>1000	n	>1000	n	>1000	n	118±23	8	>1000	n	>1000	n	>1000	n

[a]Values represent the Mean±SD from at least three independent experiments. [b]K_d values in nM units. [c]S/N represents the signal-to-noise ratio calculated in the binding assay as the ratio Total Signal/Nonspecific Signal at the K_d value. S/N was calculated at 1000 nM for K_d values over 1000 nM. [d]n indicates S/N < 2. [e]SL-S = small linker-DSX; SL-D = small linker-DY647; LL-S =long linker-SFX; LL-D = long linker-DY647; PL-S = pegylated linker-SFX; PL-D = pegylated linker-DY647; VLL-S = very long linker-SFX; VLL-D = very long linker-DY647; VLL-F = very long linker-Fluorescein.

Table 3. Determination of the Binding Affinities of Fluorescent Probes for Adrenergic Receptors Using TR-FRET Binding Assays.[a]

Id	Cpd *code*[e]	Ligand Binding TR-FRET assay											
		Adrenergic receptors											
		α_{1A}		α_{1B}		α_{1D}		α_{2A}		α_{2B}		α_{2C}	
		K_d[b]	S/N[c]	K_d	S/N	K_d	S/N	K_d	S/N	K_d	S/N	K_d	S/N
26	SL-S	308±148	4,5	>1000	n[d]	>1000	n	>1000	n	396±118	7,5	>1000	n
27	SL-D	>1000	n	>1000	n	>1000	n	>1000	n	>1000	n	>1000	n
28	LL-S	174±29	9	>1000	n	>1000	n	>1000	n	567±180	4	>1000	n
29	LL-D	121±51	2	70±35	2	>1000	n	>1000	n	>1000	n	160±76	4
30	PL-S	>1000	n	223±60	5	>1000	n	>1000	n	770±218	9	>1000	n
31	PL-D	247±44	8.5	2.4±1	2.5	>1000	n	>1000	n	127±41	6	>1000	n
32	VLL-S	131±44	3	>1000	n	>1000	n	>1000	n	432±191	6	>1000	n
33	VLL-D	>1000	n	>1000	n	>1000	n	>1000	n	72±30	2	>1000	n
34	VLL-F	>1000	2	>1000	n	>1000	n	>1000	n	180±65	6	>1000	n

[a]Values represent the Mean±SD from at least three independent experiments. [b]K_d values in nM units. [c]S/N represents the signal-to-noise ratio calculated in the binding assay as the ratio Total Signal/Nonspecific Signal at the K_d value. S/N was calculated at 1000 nM for K_d values over 1000 nM. [d]n indicates S/N < 2. [e]SL-S = small linker-SFX; SL-D = small linker-DY647; LL-S =long linker-SFX; LL-D = long linker-DY647; PL-S = pegylated linker-SFX; PL-D = pegylated linker-DY647; VLL-S = very long linker-SFX; VLL-D = very long linker-DY647; VLL-F = very long linker-Fluorescein.

Table 4. Determination of the Binding Affinities of Fluorescent Probes for Acetylcholine and Melatonin Receptors Using TR-FRET Binding Assays.[a]

		Ligand Binding TR-FRET assay													
		Acetylcholine receptors										Melatonin receptors			
	Cpd	M_1		M_2		M_3		M_4		M_5		MTN_{1A}		MTN_{1B}	
Id	*code*[e]	K_d[b]	S/N[c]	K_d	S/N	K_d	S/N	K_d	S/N	K_d	S/N	K_d	S/N	K_d	S/N
26	SL-S	>1000	n[d]	>1000	n	>1000	n	>1000	n	>1000	n	>1000	n	>1000	n
27	SL-D	>1000	n	>1000	n	>1000	n	>1000	n	>1000	n	>1000	n	>1000	n
28	LL-S	>1000	n	>1000	n	>1000	n	>1000	n	>1000	n	>1000	n	>1000	n
29	LL-D	>1000	n	>1000	n	>1000	n	>1000	n	>1000	n	>1000	n	>1000	n
30	PL-S	>1000	n	>1000	n	>1000	n	>1000	n	>1000	n	>1000	n	>1000	n
31	PL-D	>1000	n	>1000	n	>1000	n	>1000	n	>1000	n	>1000	n	>1000	n
32	VLL-S	>1000	n	>1000	n	>1000	n	>1000	n	>1000	n	>1000	n	>1000	n
33	VLL-D	>1000	n	>1000	n	>1000	n	>1000	n	>1000	n	>1000	n	>1000	n

34	VLL-F	>1000	n	>1000	n	>1000	n	>1000	n	>1000	n	>1000	n	>1000	n

[a]Values represent the Mean±SD from at least three independent experiments. [b]K_d values in nM units. [c]S/N represents the signal-to-noise ratio calculated in the binding assay as the ratio Total Signal/Nonspecific Signal at the K_d value. S/N was calculated at 1000 nM for K_d values over 1000 nM. [d]n indicates S/N < 2. [e]SL-S = small linker-SFX; SL-D = small linker-DY647; LL-S =long linker-SFX; LL-D = long linker-DY647; PL-S = pegylated linker-SFX; PL-D = pegylated linker-DY647; VLL-S = very long linker-SFX; VLL-D = very long linker-DY647; VLL-F = very long linker-Fluorescein.

Table 5. Number of receptors bound by the different fluorescent probes at sub-micromolar concentrations.

Cpd		Number of Hit Aminergic GPCRs[a]						Total
Id	Code[c]	Serotonin receptors	Dopamine receptors	Histamine receptors	Adrenergic receptors	Acetylcholine receptors	Melatonin receptors	Score[b]
26	SL-S	4/12	4/5	2/4	2/6	0/5	0/2	12/34
27	SL-D	0/12	0/5	1/4	0/6	0/5	0/2	1/34
28	LL-S	3/12	2/5	1/4	2/6	0/5	0/2	8/34
29	LL-D	6/12	4/5	1/4	3/6	0/5	0/2	14/34
30	PL-S	6/12	3/5	1/4	2/6	0/5	0/2	12/34
31	PL-D	6/12	3/5	1/4	3/6	0/5	0/2	13/34
32	VLL-S	2/12	1/5	2/4	2/6	0/5	0/2	7/34
33	VLL-D	5/12	2/5	1/4	1/6	0/5	0/2	9/34
34	VLL-F	1/12	1/5	1/4	1/6	0/5	0/2	4/34

[a]Hit aminergic GPCRs represent GPCRs for which a fluorescent ligand shows a K_d value under 1000 nM. [b]Total Score represents the addition of all hit aminergic GPCRs found during the ligand binding study for the designated fluorescent ligand. [c]SL-S = small linker-SFX; SL-D = small linker-DY647; LL-S =long linker-SFX; LL-D = long linker-DY647; PL-S = pegylated linker-SFX; PL-D = pegylated linker-DY647; VLL-S = very long linker-SFX; VLL-D = very long linker-DY647; VLL-F = very long linker-Fluorescein.

Table 6. Inhibition constants determined by competition assay with fluorescent probes as tracers and reference compounds as competitors.

	Competition Binding , pK_i (nM)[a]							
	$5HT_{2b}$ receptor							
	5-HT		Methysergide		5-CT		Asenapine	
Probes	Found	Lit.[48]	Found	Lit.[48,49]	Found	Lit.[48]	Found	Lit.[45]
30	9.07±0.05	7.87±0.04	9.41±0.09	8.2± ?[49]	7.92±0.18	6.73±0.09	9.73±0.14	9.75±0.03
31	8.81±0.04		9.04±0.11	9.44± 0.5[48]	7.62±0.12		9.4±0.04	
	$5HT_{1d}$ receptor							
	Donitriptan		Methysergide		5-HT		Asenapine	
Probes	Found	Lit.[53]	Found	Lit.[51,52]	Found	Lit.[51]	Found	Lit.[45]
29	9.46±0.22	9.32±0.09	8.50±0.11	8.08±0.10	nd	8.23±0.08	nd	nd

31	9.23±0.01		8.25±0.12		8.17±0.06		8.08±0.12	
	5-HT$_{2c}$ receptor				**5-HT$_6$ receptor**			
	Methylergonovine		Methysergide		Lisuride		SB 271046	
	Found	Lit.[48]	Found	Lit.[48]	Found	Lit.[54]	Found	Lit.[55]
26	7.82±0.05	8.34±0.06	8.60±0.12	8.60±0.05	7.71±0.11	10.9±1.8	8.06±0.22	8.92±?
	D$_4$ receptor							
	Bromocriptine		Quinpirole		Roxindole		Asenapine	
Probes	Found	Lit.[50,b]	Found	Lit.[50,b]	Found	Lit.[50,b]	Found	Lit.[45]
26	6.18±0.12	6.43±0.11	6.41±0.04	7.47±0.05	6.13±0.09	8.23±0.06	8.06±0.28	8.95±0.07
31	6.54±0.16		7.25±0.22		6.87±0.10		9.15±0.11	

[a]Values represent the Mean±SD from at least three independent experiments. Fluorescent ligands were used at the following concentrations: **30** and **31** were respectively used at 70 nM and 10 nM on 5HT$_{2b}$ receptor; **29** and **31**were respectively used at 4 nM and 17 nM on 5HT$_{1d}$ receptor; **26** was used at 10 nM on 5HT$_{2c}$, 5HT$_6$ and D$_4$ receptors; **31** was used at 78 nM on D$_4$ receptor. [b]h4.4. isoform of the D$_4$ receptor. Lit. : pK_i reported in the literature.[45, 48-55] nd = not done.

Figure 1

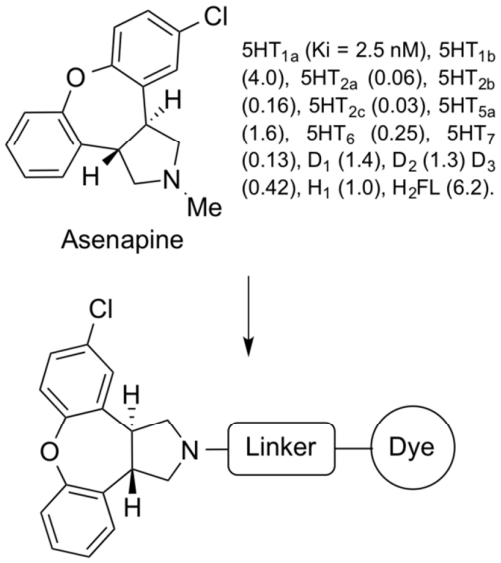


Figure 1. General strategy for the labeling of asenapine and affinity (Ki in nM) of the parent compound for a set of GPCRs.[45]

Figure 2.

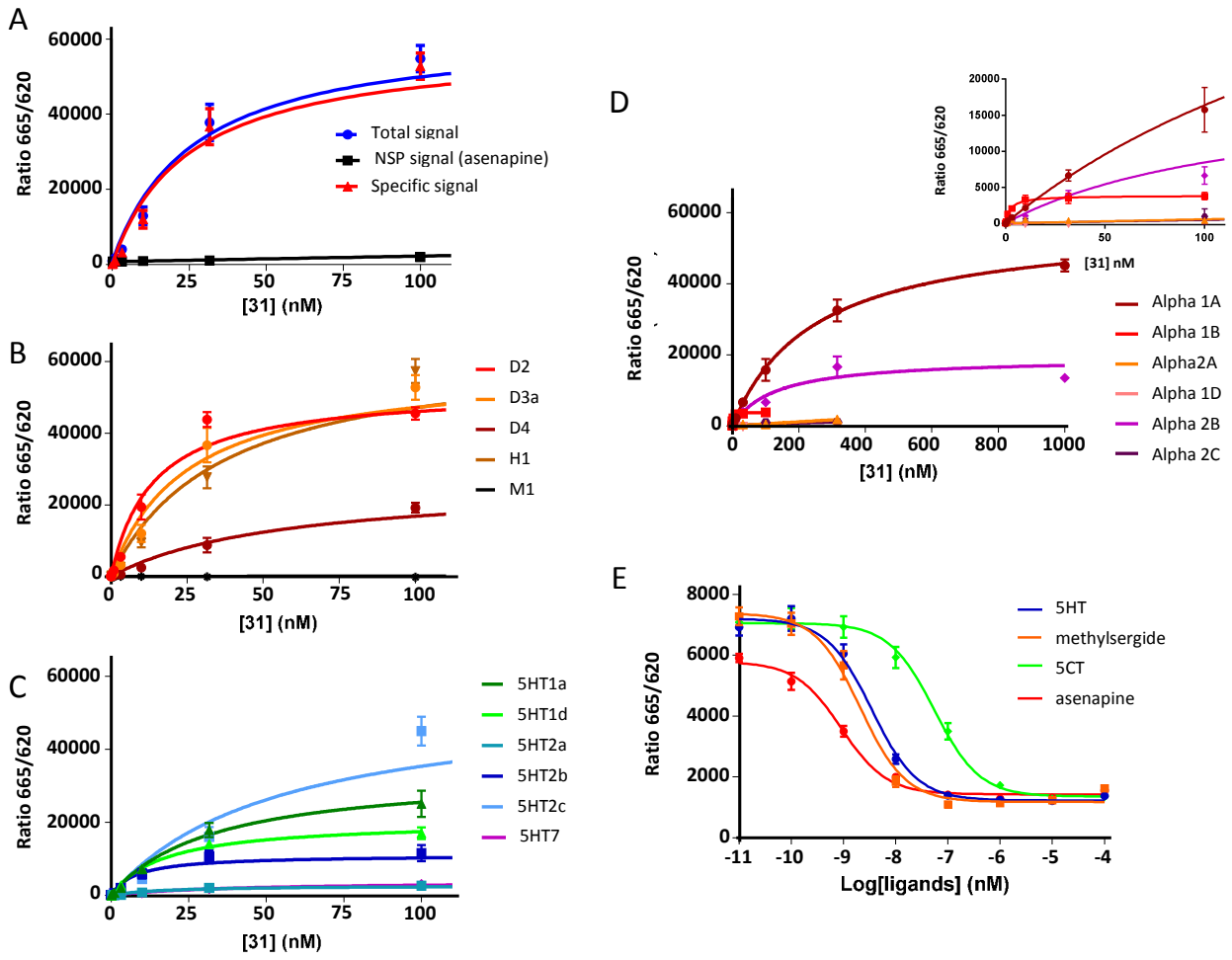
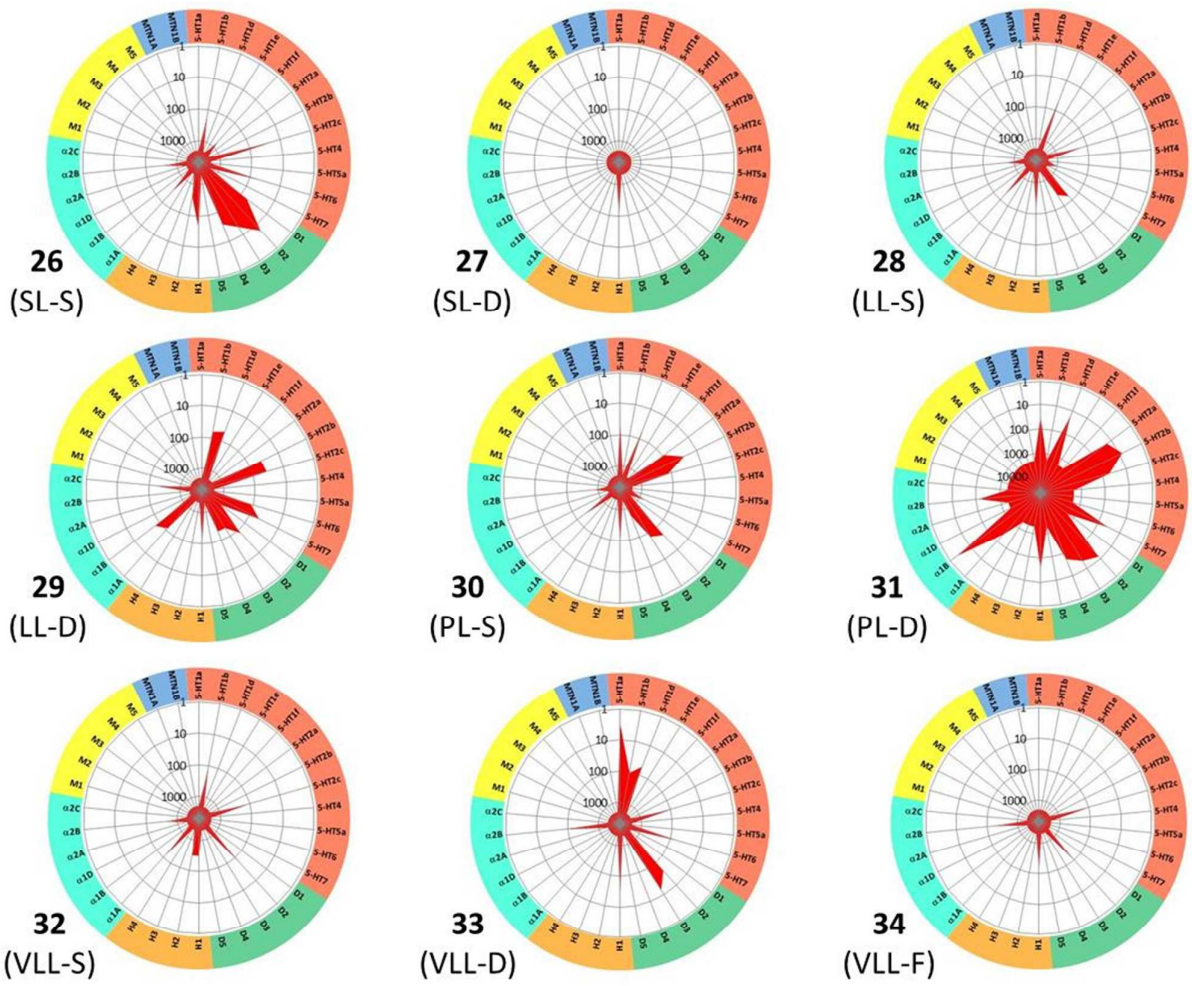
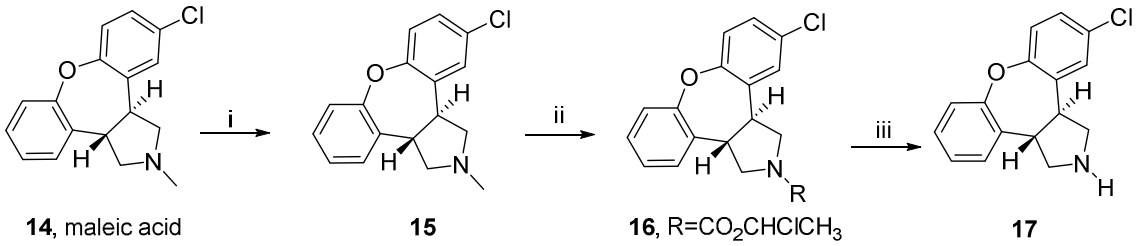

A
Ratio 665/620
[31] (nM)
Total signal
NSP signal (asenapine)
Specific signal
B
D2
D3a
D4
H1
M1
C
5HT1a
5HT1d
5HT2a
5HT2b
5HT2c
5HT7
D
[31] nM
Alpha 1A
Alpha 1B
Alpha2A
Alpha 1D
Alpha 2B
Alpha 2C
E
Log[ligands] (nM)
5HT
methylsergide
5CT
asenapine

Figure 2. Binding experiments with **31**: A, binding saturation of **31** on dopamine D_{3a} receptor. B, C, D, specific binding of **31** on various receptors belonging to the dopamine and the histamine (B), the serotonin (C), and the adrenergic (D) receptor families. E, competition binding on $5HT_{2b}$ receptor. **31** was used 17 nM. Competitors were added at a concentration ranging from 10^{-12} M to 10^{-4} M. All curves result from pooled data obtained in at least three independent experiments. Data were fitted with Prism using the one binding site subroutine in the "Binding-Competitive" procedure.

Figure 3



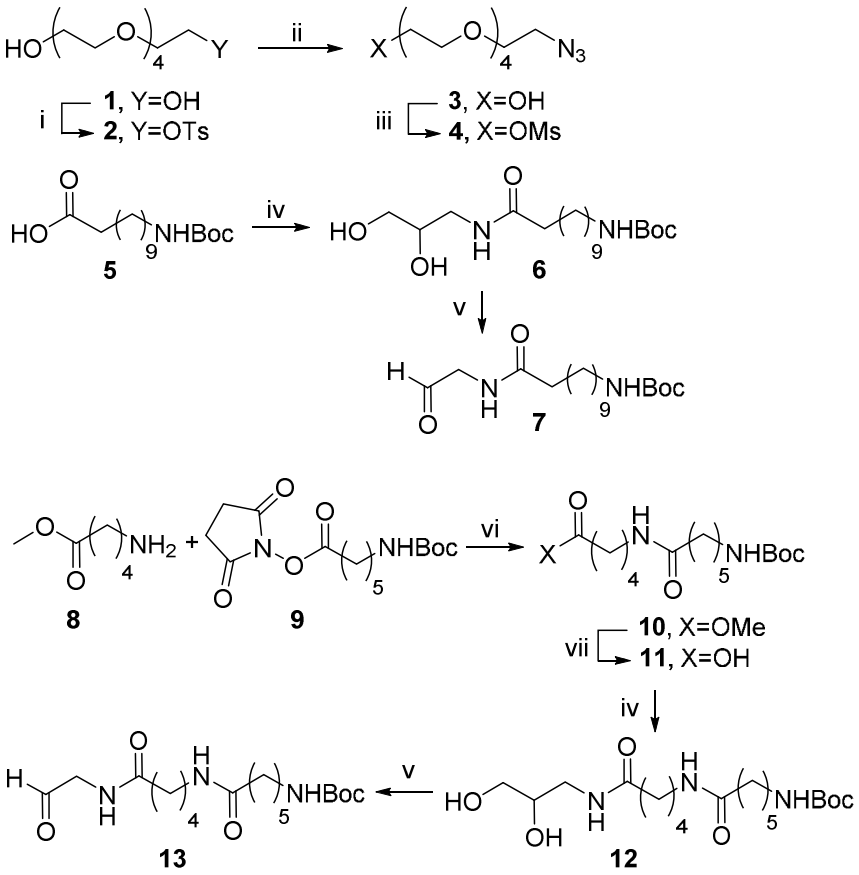
Scheme 1. Preparation of Norasenapine 17 from Asenapinium Maleate 14 (racemate)



14, maleic acid **15** **16**, R=$CO_2CHClCH_3$ **17**

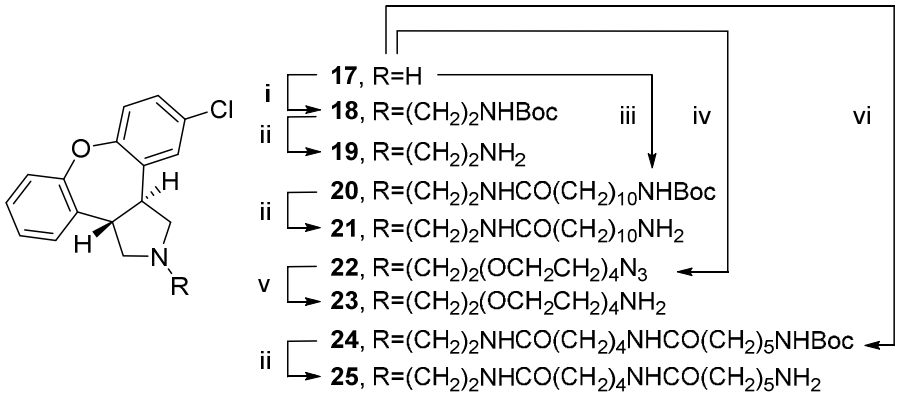
[a]Reagents : (i) PL-TBD resin, CH_2Cl_2/MeOH, overnight, rt; (ii) $ClCO_2CHClCH_3$, 1,2-dichloroethane, 100 °C, 2 h; (iii) CH_3OH, 100 °C, 2h. Global Yield (3 steps): 79%

Scheme 2. Synthesis of Linkers 4, 7, and 13



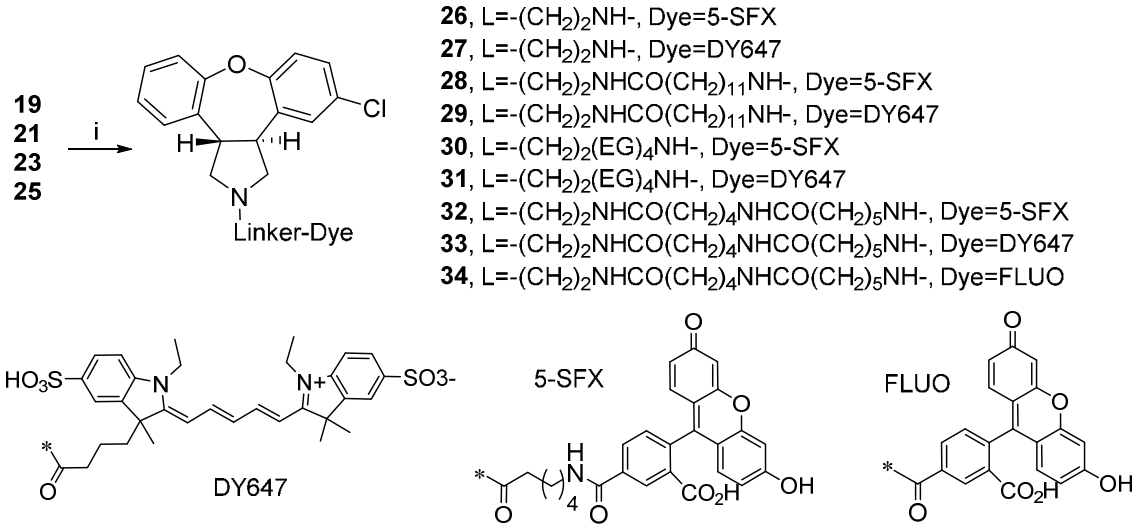
[a]Reagents : (i) TsCl, KI, Ag_2O, CH_2Cl_2, 0 °C, 15 min, 85%; (ii) NaN_3, MeCN, reflux, 12 h, 91%; (iii) MsCl, NEt_3, CH_2Cl_2, rt, 2 h, 77%; (iv) $HOCH_2CHOHCH_2NH_2$, PyBOP, *i*-Pr_2EtN, DMF/CH_2Cl_2, rt, 12 h, 93%; (v) $NaIO_4$, THF/H_2O, rt, 1 h, 100%; (vi) *i*-Pr_2EtN, DMF, rt, 4 h, 82%; (vii) Aqueous LiOH, THF, rt, 12 h, 94%.

Scheme 3. Synthesis of Amines 19, 21, 23, and 25 from Norasenapine 17 (racemates)



[a]Reagents: (i) $BocNHCH_2$-CHO, $NaBH_3CN$, MeOH, rt, 2 h, 96%; (ii) TFA, CH_2Cl_2, rt, 2 h, 65-98%; (iii) **7**, $NaBH_3CN$, MeOH, rt, 2 h, 79%; (iv) **4**, K_2CO_3, CH_3CN, rt, 12 h, then 50 °C, sonication, 2 h, 47%; (v) PS-PPh_3, THF/H_2O, rt, 12 h, 95%, (vi) **13**, $NaBH_3CN$, MeOH, rt, 2 h, 64%.

Scheme 4. Synthesis of Fluorescent Compounds 26-34 (racemates) for TR-FRET Applications.[a]



[a]Reagents: (i) Dye succinimidyl ester, *i*-Pr_2EtN, DMSO, rt, 4 h, 30-40%.

FIGURE CAPTIONS

Figure 1. General strategy for the labeling of asenapine and affinity (Ki in nM) of the parent compound for a set of GPCRs.

Figure 2. Binding experiments with **31**: A, binding saturation of **31** on dopamine D_{3a} receptor. B, C, D, specific binding of **31** on various receptors belonging to the dopamine and the histamine (B), the serotonin (C), and the adrenergic (D) receptor families. E, competition binding on 5-HT_{2b} receptor. **31** was used at17 nM. Competitors were added at a concentration ranging from 10^{-12} M to 10^{-4} M. All curves result from pooled data obtained in at least three independent experiments. Data were fitted with Prism using the one binding site subroutine in the "Binding- Competitive" procedure.

Figure 3. Web of affinity. Schematic representation of the affinity profiles (*Kd* in nM) of the different fluorescent probes for a set of 34 class A GPCRs. Compounds **26-34** ligands are also identified in a coded manner as follows: [e]SL-S = small linker-SFX; SL-D = small linker-DY647; LL-S =long linker-SFX; LL-D = long linker-DY647; PL-S = pegylated linker-SFX; PL-D = pegylated linker-DY647; VLL-S = very long linker-SFX; VLL-D = very long linker-DY647; VLL-F = very long linker-Fluorescein.

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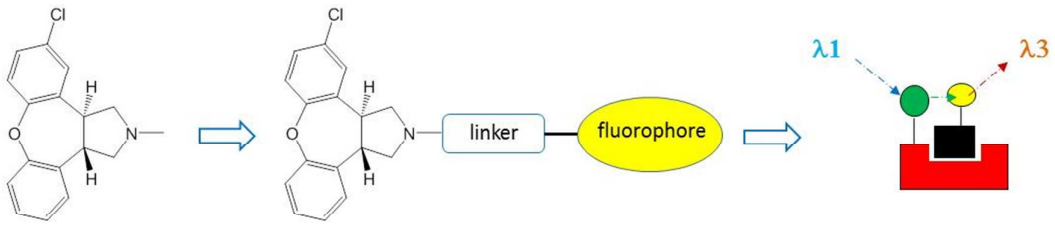

Asenapine

Nanomolar affinity for class A GPCRs

Fluorescent asenapine derivatives

High affinity fluorescent probes for 14 class A GPCRs

Suitable for Class A GPCR TR-FRET binding assays